Cytosolic Protein Quality Control of the Orphan Protein Fas2, a Novel Physiological Substrate of the E3 Ligase Ubr1

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Hiermit versichere ich, dass ich diese Arbeit selbst verfasst und dabei keine anderen als die angegeben Quellen und Hilfsmittel verwendet habe.

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Mario Scazzari

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List of abbreviations

Å	Ångström
AAA	ATPases associated with diverse cellular activities
ADP	Adenosine-5'-diphosphate
Amp	Ampicillin
APC	Anaphase promoting complex
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
bp(s)	base pair(s)
BSA	Bovine serum albumin
CM	Complete minimal media
CM+FA	Complete minimal media + fatty acid
CPY	Carboxypeptidase Y
CPY*	mutated carboxypeptidase Y (*prc1-1*)
C-terminal	Carboxy terminal
CytoQC	Cytosolic protein quality control
Da	Dalton
ddH_2O	double distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleosid triphosphate
ΔssCG*	ΔssCPY*-GFP (Δss = lacking signal sequence for ER-import)
ΔssCL*	ΔssCPY*-Leu2-myc (Δss = lacking signal sequence for ER-import)
ΔssPrA	Proteinase A, lacking signal sequence (Δss) for ER-import
DTT	Dithiothreitol
DUB	Deubiquitinating enzyme
ECL	Enhanced chemiluminescence
E . coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
FAS	Fatty acid synthase
Fig	Figure
Flag	Epitope tag; a hydrophilic and immunogenic octapeptide
G6PDH	Glucose-6-phosphate dehydrogenase
GFP	Green fluorescent protein
Glc	Glucose
HA	Hemagglutinin
HECT	Homologous to the E6-AP carboxyl terminus
HRPO	Horseradish peroxidase
Hsp	Heat shock protein
IPOD	Insoluble protein deposit
JUNQ	Juxtanuclear quality control compartment
Kb(s)	Kilobase(s)
KDa	Kilodalton
LB-media	Lysogeny broth media
M	molar
mbar	millibar

min	minute(s)
ml	milliliter
mM	millimolar
MW	Molecular weight
NLS	Nuclear localization sequence
µl	microliter
µCi	microcurie
NBD	Nucleotide binding domain
NEF	Nucleotide exchange factor
N-terminal	Amino terminal
OD_{600}	Optical density at 600 nanometer wavelength
ODC	Ornithine decarboxylase
o/n	over night
PAGE	Polyacrylamide gel electrophoresis
P_{GAL1}	Galactose-inducible promoter
PEG	Polyethylene glycol
PGK	3-Phosphoglyceratkinase
PQC	Protein quality control
RING	Really interesting new gene
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
RT	Room temperature
S	Svedberg
S. cerevisiae	*Saccharomyces cerevisiae*
TAP	Tandem affinity purification
TCA	Trichloroacetic acid
TEMED	*N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine
Tris	Tris(hydroxymethyl)aminomethane
ts	Temperature sensitive
Uba	Ubiquitin activating enzyme
Ubc	Ubiquitin conjugating enzyme
UPS	Ubiquitin proteasome system
V5	epitope tag; represents amino acid residues 95-108 of RNA polymerase alpha subunit of simina virus 5
v/v, w/v	Volume per volume, weight per volume
VHL	von Hippel-Lindau tumor suppressor
WT	Wild type
YCM+FA	Complete minimal media + yeast extract + fatty acid
YPD	Yeast peptone dextrose
YPD+FA	Yeast peptone dextrose + fatty acid
YPGal	Yeast peptone galactose
YPGal+FA	Yeast peptone galactose + fatty acid

1 Abstract

Cellular protein quality control (PQC) monitors the proper folding of polypeptides, assembly of protein subunits into protein complexes as well as the delivery of terminally misfolded proteins to degradation. The components of PQC known best at the moment are molecular chaperones and the ubiquitin proteasome system. In contrast to the well-described protein quality control system of the endoplasmic reticulum (ERAD), less is known about how misfolded proteins in the cytosol are recognized and degraded. The cytosolic fatty acid synthase complex (FAS) of *Saccharomyces cerevisiae*, which is composed of six Fas1- and six Fas2-subunits, is rather stable to proteolysis *in vivo*. In the absence of the Fas1 subunit (*FAS1* deletion strain) the remaining Fas2 subunit becomes an orphan protein which is proteolytically unstable and is targeted to the 26S proteasome for degradation (Egner et al, 1993). In my work, I used the orphan Fas2 protein as object of investigation in order to identify new cellular components that are involved in the recognition and degradation of a natural unassembled protein subunit in *S. cerevisiae*. In addition, it was elucidated how these newly identified factors act in the quality control process of a naturally occurring orphan protein. Due to previous reports (Heck et al, 2010; Prasad et al, 2010) showing that some cytosolic misfolded proteins are imported into the nucleus for proteasomal degradation the cellular localization of orphan Fas2 was determined. Using laser-scanning microscopy it could be shown that C-terminally EGFP-tagged (enhanced green fluorescent protein) orphan Fas2 is localized to the cytosol, thus representing a potential substrate for the cytosolic quality control system (CytoQC). Furthermore, glycerol step density gradient centrifugation experiments revealed that the majority of the orphan Fas2 proteins are organized in high molecular assembly intermediates, which consist mostly of Fas2 homohexamers. By using the thermosensitive *ssa1-45* mutant carrying in addition the gene deletions of *SSA2, SSA3* and *SSA4*, it could be shown that the proteasomal degradation of the orphan protein is dependent on the Hsp70 chaperone Ssa1. It is likely that Ssa1 is required for keeping orphan Fas2 soluble. All members of the Hsp90-, Hsp100-, and Hsp110-chaperone family as well as the small heat shock proteins Hsp26 and Hsp42 were shown to have no effect on the degradation of orphan Fas2. Selected members of the Hsp40 chaperone family, including Apj1, Xdj1 and even Ydj1 also did not show a significant influence on the Ssa1-dependent elimination of the substrate. To prove whether other components of

the UPS than the proteasome are required for degradation of orphan Fas2 different E2- and E3 gene deletion mutants were analyzed. It was found that the elimination of orphan Fas2 is strongly delayed in a strain carrying a *UBC2 UBC4* double deletion. As single deletions of *UBC2* and *UBC4* have no significant effect on the turnover of the substrate, it can be assumed that these E2 enzymes have complementing functions in the degradation process of orphan Fas2. In a search for the responsible E3 ubiquitin ligase(s) required for orphan Fas2 degradation the E3 RING ligase Ubr1 was identified. Deletion of *UBR1* leads to a strongly delayed degradation of orphan Fas2. The expression of an Ubr1 RING mutant (C1220S) or of an Ubr1 type-1 N-end rule mutant (D176) from a high-copy plasmid in the *UBR1* deletion strain cannot complement the strongly delayed degradation of orphan Fas2. In contrast, the stabilization of the orphan protein in the *UBR1* deletion strain is reversed, when the same strain harbours a high-copy plasmid expressing wild type Ubr1 or an Ubr1 type-2 N-end rule mutant (P406S) or even a cytosolically-located version of the nuclear E3 RING ligase San1 due to deletion of the nuclear localization sequence. Interaction studies revealed that the E3 RING ligase Ubr1 is physically associated with orphan Fas2. In addition, it was found that Ubr1 mutants harbouring either a defect RING domain or a defect in one of the N-end rule substrate-binding sites (type-1 or type-2) were still able to physically interact with orphan Fas2. Further studies showed that the physical association of orphan Fas2 and Ubr1 remains stable in the conditional *ssa1-45* mutant carrying in addition deletions of *SSA2* to *SSA4*. This indicates that already E3-bound orphan Fas2 may not require a functional peptide-binding domain of Ssa1 to maintain the physical contact to Ubr1. Finally, the AAA-ATPase Cdc48 was identified to be necessary for the elimination process of orphan Fas2. Cdc48 may function in the dissociation process of orphan Fas2 assembly intermediates, which mainly consist of Fas2-homohexamers.

2 Zusammenfassung

Die zelluläre Proteinqualitätskontrolle überwacht die korrekte Faltung von Polypeptiden, die Assemblierung von Proteinuntereinheiten in Proteinkomplexen sowie den Abbau von irreversibel fehlgefalteten Proteinen. Die molekularen Chaperone und das Ubiquitin-Proteasom-System (UPS) sind die derzeit am Besten bekanntesten Komponenten der zellulären Proteinqualitätskontrolle. Im Gegensatz zu dem gut untersuchten Proteinqualitätskontrollsystem des Endoplasmatischen Retikulums (ERAD) ist relativ wenig über die Erkennung und den Abbau von fehlgefalteten Proteinen im Cytosol bekannt. Die cytosolische Fettsäuresynthase (FAS) von *Saccharomyces cerevisae*, welche aus sechs Fas1- und sechs Fas2-Proteinuntereinheiten besteht, ist ein *in vivo* proteolytisch stabiler Enzymkomplex. In Abwesenheit der Fas1 Untereinheit (*FAS1* Deletion) wird die verbleibende Fas2 Untereinheit zu einem Waisenprotein (Orphanprotein), welches proteolytisch instabil ist und durch das 26S Proteasom abgebaut wird (Egner et al, 1993). In meiner Arbeit habe ich Orphan Fas2 als Untersuchungsobjekt benutzt, um weitere zelluläre Komponenten der cytosolischen Proteinqualitätskontrolle zu identifizieren, die an der Erkennung und am Abbau einer natürlichen und nicht-assemblierten Proteinuntereinheit in *S. cerevisiae* beteiligt sind. Des Weiteren sollten die genauen Funktionen der neu entdeckten Komponenten im Qualitätskontrollprozess dieses natürlich vorkommenden Orphanproteins bestimmt werden.

Aufgrund vorheriger Berichte (Heck et al, 2010; Prasad et al, 2010), welche zeigen, dass der proteasomale Abbau einiger cytosolischer, fehlgefalteter Proteine im Zellkern stattfindet wurde zunächst die zelluläre Lokalisation von Orphan Fas2 bestimmt. Mittels Laser Scanning Mikroskopie konnte nachgewiesen werden, dass Orphan Fas2-EGFP (enhanced green fluorescent protein) gleichmäßig im Cytosol der Bäckerhefe lokalisiert ist und somit ein potentielles Substrat der Cytosolischen Proteinqualitätskontrolle (CytoQC) darstellt. Unter Verwendung der Dichtegradientenzentrifugation stellte sich heraus, dass die meisten Orphan Fas2 Proteine in hochmolekularen Assemblierungs-intermediaten vorliegen, die wiederum hauptsächlich aus Fas2 Homohexameren bestehen. Mit Hilfe der temperatursensitiven *ssa1-45* Mutante, in der zusätzlich alle weiteren *SSA* Gene (*SSA2*, *SSA3*, *SSA4*) deletiert sind, konnte gezeigt werden, dass der proteasomale Abbau von Orphan Fas2 vom Hsp70 Chaperone Ssa1 abhängig ist. Man kann davon ausgehen, dass Ssa1 bei der Aufrechterhaltung der Löslichkeit von Orphan

Fas2 eine wichtige Rolle spielt. Alle Mitglieder der Hsp90-, Hsp100- und der Hsp110-Chaperone Familie sowie die kleinen Hitzeschockproteine, Hsp26 und Hsp42, haben dagegen keinen Einfluss auf den Abbau des Orphan Substrats. Ausgewählte Mitglieder der Hsp40 Chaperone Familie, einschließlich Apj1, Xdj1 und sogar Ydj1, haben ebenso keinen Effekt auf die Ssa1-abhängige Eliminierung von Orphan Fas2. Um zu prüfen ob neben dem Proteasom noch weitere Komponenten des UPS am Abbau des Fas2 Orphanproteins beteiligt sind wurden diverse E2 und E3 Deletionsmutanten getestet. Der Abbau von Orphan Fas2 ist in einem *UBC2 UBC4* Doppeldeletionsstamm stark verzögert. Da die jeweiligen Einzeldeletionen von *UBC2* und *UBC4* keinen signifikanten Effekt auf den Abbau des Substrats haben, kann man davon ausgehen, dass diese beiden E2 Enzyme komplementierende Funktionen im Bezug auf den Abbau von Orphan Fas2 besitzen. Bei der Suche nach entsprechenden E3 Ubiquitin Ligasen, die an der Qualitätskontrolle des Fas2 Orphanproteins beteiligt sind, konnte die E3 RING Ligase Ubr1 identifiziert werden. Eine Deletion von *UBR1* führt zu einem stark verzögerten Abbau des Substrats. Die Expression einer Ubr1 RING Mutante (C1220S) oder einer Ubr1 Type-1 N-end Rule Mutante (D176E) von einem High-copy Plasmid in einem *UBR1* Deletionsstamm hatte keinen Einfluss auf den stark verlangsamten Abbau von Orphan Fas2. Im Gegensatz dazu wird die Stabilisierung von Orphan Fas2 in einem *UBR1* Deletionsstamm wieder aufgehoben wenn dieser gleichzeitig ein High-copy Plasmid enthält welches entweder Wildtyp Ubr1, eine Ubr1 Type-2 N-end Rule Mutante (P406S) oder eine cytosolisch-lokalisierte Variante der nukleären E3 RING Ligase San1 (San1 ohne Kernlokalisierungssequenz) exprimiert. Interaktionsstudien haben gezeigt, dass die E3 RING Ligase Ubr1 mit Orphan Fas2 physikalisch assoziiert ist. Es hat sich weiterhin herausgestellt, dass Ubr1 Mutanten, die entweder eine defekte RING Domäne (C1220S) oder eine Mutation in einer der N-End Rule Substratbindestellen (D176E oder P406S) besitzen, immer noch in der Lage sind mit Orphan Fas2 zu interagieren. Ergebnisse weiterer Bindungsstudien konnten außerdem darlegen, dass zumindest eine bereits bestehende physikalische Interaktion zwischen Orphan Fas2 und Ubr1 in der temperatursensitiven *ssa1-45* Mutante, in der zusätzlich alle weiteren *SSA* Gene (*SSA2*, *SSA3*, *SSA4*) deletiert sind, bestehen bleibt. Schließlich wurde die AAA-ATPase Cdc48 als ein weiterer wichtiger Faktor für den Abbau von Orphan Fas2 identifiziert. Cdc48 ist wahrscheinlich an der Dissoziation von Fas2 Assemblierungsintermediaten, welche hauptsächlich aus Fas2 Homohexameren bestehen, beteiligt.

3 Introduction

3.1 Folding and misfolding of cytosolic proteins

Proposed by the Swedish scientist Jöns Jacob Berzelsius in 1838 and first published by the Dutch chemist Gerardus Johannes Mulder the term "protein" was established in 1839 (Mulder, 1839); (from ancient greek: protos, which stands for "first class" or "the most important"). Proteins are one of the most important macromolecules in living cells because they are involved in a large number of biological processes. Many proteins act as enzymes catalyzing biochemical reactions. Other proteins are involved in cell signalling, cell adhesion, cell cycle or are components of the immune system. A protein is build up from a set of 20 biogenous amino acids joined by a peptide bond between the alpha carboxyl and the alpha amino groups of adjacent amino acid residues (Stryer, 1996). Proteins are first synthesized in form of a polypeptide chain translated from messenger RNA by ribosomes. The basis for a functional protein is its unique native three-dimensional structure, which is characteristic for each protein (Dill et al, 1995). The process in which an unfolded linear polypeptide chain folds to a defined three-dimensional structure is called protein folding and is determined by its amino acid sequence (Anfinsen et al, 1961). Folding of proteins takes place in the cytosol, the endoplasmic reticulum (ER), mitochondria and the secretory pathway. Due to the highly crowded molecular environment and the fact that nascent polypeptides tend to aggregate because of their exposed hydrophobic regions, nascent polypeptides need special helper proteins for proper folding. These helpers include molecular chaperones preventing the aggregation of nascent polypeptides, thus stabilizing their non-native conformation and assist their proper folding (Bukau & Horwich, 1998; Hartl & Hayer-Hartl, 2002). Additional important functions of molecular chaperones are the refolding of unfolded- or misfolded proteins, the prevention and disaggregation of protein aggregates or the guidance of terminally misfolded- and unwanted proteins to cellular protein degradation machinery (Esser et al, 2004; Liberek et al, 2008; Wickner et al, 1999). Degradation of cellular proteins is executed mainly by two intracellular proteolytic pathways including the lysosomal (vacuole)–system and the ubiquitin proteasome system. The components of cellular protein quality control known best at the moment are the molecular chaperones and the ubiquitin proteasome system. Protein quality control systems have been identified in the ER, the secretory pathway, the nucleus, in

mitochondria and also in the cytosol (Baker et al, 2011; Buchberger et al, 2010; Stolz & Wolf, 2010; Vembar & Brodsky, 2008; Voos, 2009). It is supposed that cellular PQC is responsible for certain activities in living cells (Anelli & Sitia, 2008):
(1) Promoting the folding of proteins into their correct three-dimensional structure, (2) To keep precursor proteins in a suitable state for their maturation, (3) To promote correct assembly of protein complexes, (4) Detection of proteins that are unable to fold properly / misassembled proteins, (5) Preventing protein aggregation, (6) Protein degradation of terminally misfolded proteins in order to reduce the risk of proteotoxicity. Actually, the preferred model of cytosolic protein quality control in eukaryotic cells includes the active involvement of molecular chaperones on the degradation of misfolded proteins (Esser et al, 2004; Hohfeld et al, 2001). The decision to refold or to initiate the degradation of a misfolded protein is determined by chaperone–associated cofactors. The association of a chaperone with a "folding factor" induce and maintain the protein folding process, whereas the interaction of a chaperone with a "degradation factor" leads to the recruitment of UPS components and finally to the degradation of the unfolded protein (Arndt et al, 2007; McDonough & Patterson, 2003; Wickner et al, 1999).

3.1.1 The role of different chaperone families in protein folding and misfolding

In natural science the term chaperone describes a protein that transiently interacts and stabilizes an unstable conformer of another protein, thus facilitating its folding, oligomeric assembly, intracellular transport or proteolytic degradation, either singly or with the help of other cofactors (Hendrick & Hartl, 1993) .
In the eukaryotic cytosol the process of protein folding is mediated and controlled by a network of different molecular chaperones. The association of misfolded and aggregated proteins with neurodegenerative disorders underscores the importance of cellular chaperones in promoting and retaining the native conformation of proteins in the cytosol and other cell compartments. Parkinson's disease and adult-onset Huntington's disease are associated with the formation and especially the accumulation of intracellular aggregation–prone proteins predominantly in the cytosol (Dobson, 2003; Selkoe, 2003). In addition there are also other cellular proteins that are associated with the appearance of such neurodegenerative diseases (described later in chapter3.2.2.1).

The Hsp70 chaperone family

Hsp70s are ubiquitous proteins found in eukaryotic cells, eubacteria and in some archaea. They assist folding of newly synthesized proteins, are involved in the translocation of unfolded polypeptides across intracellular membranes and mediate the heat shock response (Hartl, 1996; Hartl & Hayer-Hartl, 2002). Further studies have shown that Hsp70 chaperones also play an important role in the delivery of misfolded cytosolic proteins to the proteasome (McClellan et al, 2005; Park et al, 2007). The recognition of exposed hydrophobic regions of the newly synthesized polypeptides is mediated by the internal peptide–binding domain of Hsp70 (Bukau & Horwich, 1998). To regulate binding and release of the polypeptide chain Hsp70 contains a C-terminal EEVD motif that is proposed to act as a lid for the peptide–binding domain. The mechanistic lid opening and closing is regulated by binding and hydrolysis of ATP performed by the N–terminal ATPase domain of Hsp70 (Figure 1b). The lid uncovers the peptide-binding pocket resulting in a low binding affinity to the polypeptide, when Hsp70 is present in the ATP-bound state, whereas after ATP hydrolysis the lid of ADP-bound Hsp70 covers the peptide-binding pocket resulting in an high binding affinity to the polypeptide chain (Figure 1a) (Kampinga & Craig, 2010). Two of the first components getting in contact with a nascent polypeptide at the ribosome are the Ssb proteins, Ssb1 and Ssb2. They constitute one subfamily of cytosolic Hsp70 chaperones. It is assumed that the two Ssb proteins facilitate the folding of nascent polypeptides at the ribosome (Gautschi et al, 2002; Pfund et al, 1998).

The *SSA* genes (*SSA1-4*) encode proteins (Ssa1-4) belonging to the second subclass of the yeast cytosolic Hsp70 chaperones. Ssa proteins are involved in variety of cellular processes, including protein folding, protein transport across membranes and proteasome mediated protein degradation (Hartl & Hayer-Hartl, 2002; McClellan et al, 2005; Park et al, 2007; Young et al, 2003). The importance of the SSA chaperone subfamily is underscored by the fact that expression of at least one *SSA* gene is essential for cell viability suggesting redundant functions for these molecular chaperones (Werner-Washburne et al, 1987). Additional members of yeast cytosolic Hsp70 chaperone family are Ssz1, Sse1 and Sse2. As part of the ribosome-associated complex (RAC), which includes also the Ssb proteins, the Ssz1 chaperone participate to the folding of nascent polypeptides (Gautschi et al, 2002). The function of Sse1 and Sse2 is described in the next section.

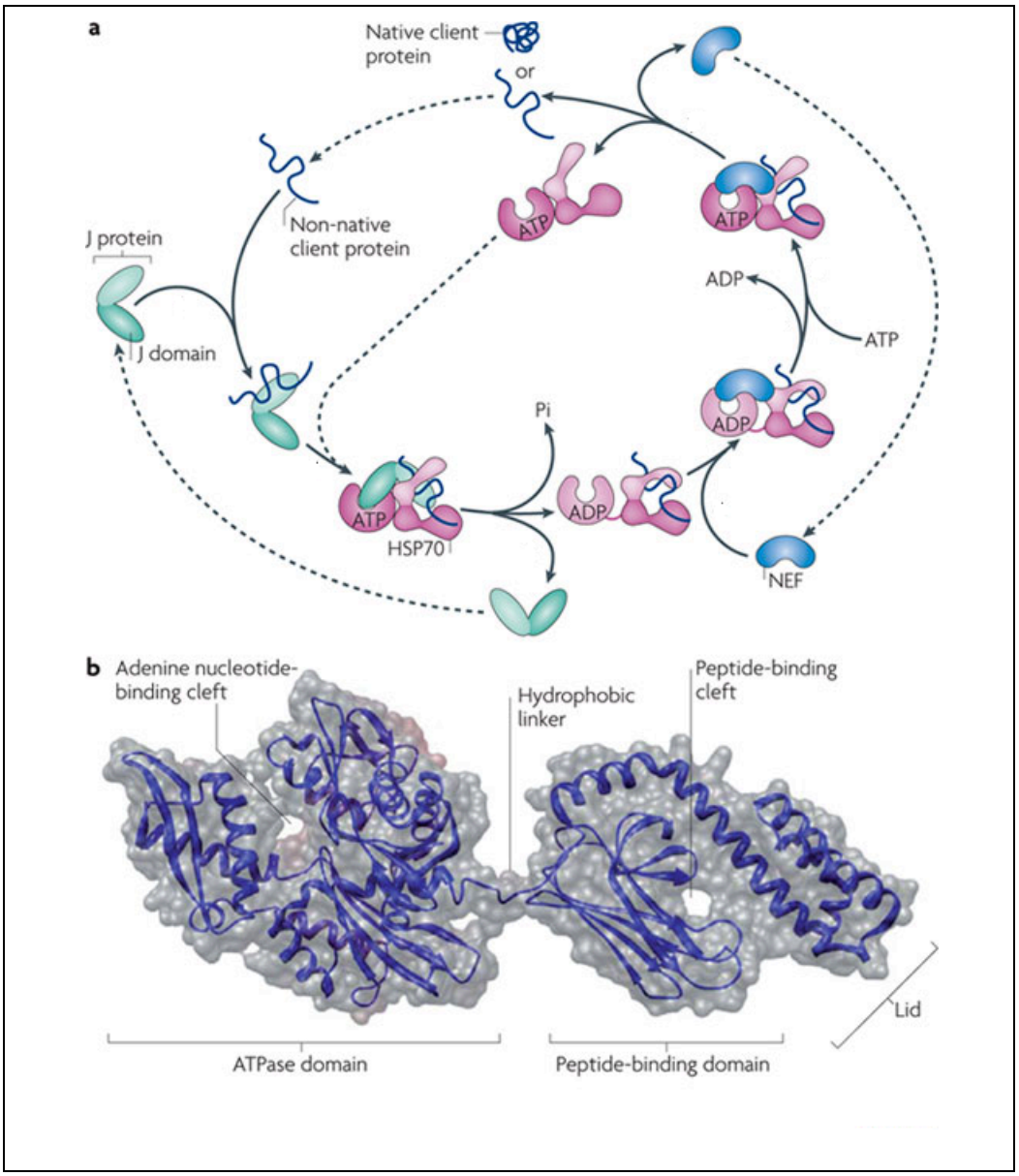

Figure 1. Hsp70 chaperone cycle during protein folding and Hsp70 structure.

a. Hsp70 ATPase cycle (reproduced by Kampinga and Craig, 2010). The non-native client protein is delivered by a J-protein (Hsp40) to an ATP-bound Hsp70. Upon lid opening Hsp70 binds the client protein via its peptide-binding cleft. The J-protein stimulates the ATPase domain of Hsp70 resulting in the hydrolysis of the bound ATP molecule. Consequently, the lid closes the peptide-binding cleft thereby trapping the client protein. The Hsp70-ADP-substrate complex releases the J-protein and the trapped substrate has a limited time to fold properly. Then, a nucleotide exchange factor (NEF) binds to the Hsp70-ADP-substrate complex and exchanges ADP with ATP. The properly folded client protein is released and Hsp70-ATP is ready for a new cycle. **b.** Hsp70 structure. The N-terminal ATPase domain and the peptide-binding domain, which covers the peptide-binding cleft, are physically connected via a hydrophobic linker. The lid, located at the C-terminus, is shown in the closed state.

Hsp70 cofactors: Hsp40 chaperones and nucleotide exchange factors

The process of ATP binding and hydrolysis by the ATPase domain of Hsp70 is very inefficient. Therefore, so-called Hsp40 chaperones make this physiological relevant process of ATP binding and hydrolysis on Hsp70 efficient as Hsp70 itself is unable to stimulate the switch to the ADP-bound state (Bukau & Horwich, 1998; Frydman & Hohfeld, 1997). A nucleotide exchange factor (NEF), a second type of Hsp70 cofactor, is required to release the polypeptide chain again. A NEF stimulates the dissociation of ADP and rebinding of ATP on Hsp70. Thereby the conformation state of the lid switches from "close" to "open" and the polypeptide is released (Alberti et al, 2003; Frydman & Hohfeld, 1997; Hohfeld et al, 1995). Thus, for the proper function of Hsp70 chaperones in protein (re)-folding, they need the assistance of two types of co-factors, an Hsp40 chaperone responsible for stimulation of Hsp70 ATPase activity and specific substrate capturing as well as a NEF important for ADP dissociation and substrate release.

In *S. cerevisiae* 22 members of the Hsp40 chaperone family have been identified so far, including 13 candidates localized to the cytosol (Sahi & Craig, 2007). All Hsp40 chaperones possess a J domain (which was described first for the Hsp40 DnaJ in *E. coli*) crucial for the physical interaction with the ATPase domain of an Hsp70 chaperone and thus stimulating the Hsp70 ATPase activity. Variation in this J-domain provides specificity for Hsp70-Hsp40 interaction (Hennessy et al, 2000; Karzai & McMacken, 1996). The most abundant member of cytosolic Hsp40 is Ydj1, which is involved in a variety of processes in eukaryotic cells, including folding and refolding of proteins (Lu & Cyr, 1998), suppression and rescue of protein aggregates (Glover & Lindquist, 1998) and even in proteasome mediated degradation of proteins (Lee et al, 1996; Park et al, 2007; Prasad et al, 2010). Recent studies have revealed that Hsp40 chaperones can function without or with binding to the client protein during the Hsp70 reaction cycle (Kampinga & Craig, 2010). In the first case, only Hsp70 binds directly the client substrate. The Hsp40 is either untethered or tethered to a specific location in the cell. Upon binding of the client protein to Hsp70 the J-domain of the Hsp40 stimulates the ATPase activity of the client-bound Hsp70. In the second case, an Hsp40 chaperone with a client protein-binding domain can function in two different ways. On one side the Hsp40 chaperone binds the client protein via its client protein-binding binding domain first and transfers it to the Hsp70 or, Hsp40 binds directly to the client-bound Hsp70. In this situation, despite the existence of a client protein-binding domain the Hsp40 chaperone only stimulates the ATPase activity of the Hsp70 chaperone. The proteins

Fes1, Snl1 and the two Hsp110 chaperones Sse1 and Sse2 are NEFs of the cytosolic Hsp70 chaperones (Andreasson et al, 2008a; Andreasson et al, 2008b; Kabani et al, 2002; Rampelt et al, 2011). Due to sequence similarities the Hsp 110 chaperone family is a subclass of the Hsp70 family. Addition to their nucleotide exchange function (Dragovic et al, 2006; Raviol et al, 2006; Shaner et al, 2005) Sse chaperones are able to bind unfolded polypeptides and act as "holdases", thus preventing misfolding and aggregation of proteins (Easton et al, 2000) The importance of the Hsp110 chaperones for *S. cerevisiae* is underscored by the fact that double deletion of *SSE1* and *SSE2* is lethal (Raviol et al, 2006).

The Hsp90 chaperone family

It has been shown that Sse1 acts also as a co-chaperone of the most abundant chaperones in the eukaryotic cytosol, the Hsp90. This chaperone family consists of two members; Hsc82 and Hsp82 that share 97% sequence identity. Expression of Hsp82 is induced under heat shock or other stress conditions, whereas Hsc82 is permanently expressed at high levels and slightly induced by heat shock. Deletion of one of the Hsp90 genes (*HSC82* or *HSP82*) can be tolerated by *S. cerevisiae*, but deletion of both genes is lethal. This chaperone family contributes to folding- and refolding of proteins into their native conformation, thus preventing protein aggregation (Nathan et al, 1997). Besides their important role in protein folding, the activity of Hsp90s is also required for activation of many signalling- and regulatory proteins in the eukaryotic cell (Picard, 2002). An Hsp90 protein is composed of four structural domains: N-terminal ATPase domain, a charged linker region, a middle region (M-domain) followed by a 12 KDa C-terminal dimerization domain (Pearl & Prodromou, 2001). The C-terminus of eukaryotic Hsp90 includes further a so-called MEEVD motif, which serves as the major interaction site of several co-chaperones, e.g. Sti1, Sba1, Aha1 and Cdc37. They are able to accelerate or to slow down certain steps in the ATPase cycle of the Hsp90 chaperone. In order to become functional, Hsp90 must at form a homodimer in which the major contact sites are located to the C-terminus (Wayne & Bolon, 2007). The binding of ATP to each of the N-terminal ATPase domains of the Hsp90 homodimer induces a conformational change leading to the closure of the ATP lids. At the same time an Hsp90 client protein (e.g. unfolded- or misfolded protein) is bound to some C-terminally exposed hydrophobic residues. The closed state of the Hsp90 dimer (molecular clamp) is formed when the N-terminal domains have been dimerized. In this

closed state the Hsp90 dimer holds the client protein (that is why Hsp90 is also called „holdase"), allowing it to fold into the correct three-dimensional structure. In the closed state ATP hydrolysis is driven by the N-terminal ATPase followed by a conformational change from the closed- to an open state, releasing ADP, P_i and the properly folded client protein (Wandinger et al, 2008). The ATPase activity, and thus the function of Hsp90, can be modulated by several Hsp90 co-chaperones (e.g. Sti1, Sba1, Aha1 and Cdc37) or by some natural compounds (e.g. geldanamycin or radicicol). These highly specific drugs strongly decrease the activity of the ATPase, thus blocking the maturation of client proteins and eventually resulting in their degradation (Mabjeesh et al, 2002; Sultana et al, 2012; Whitesell et al, 1994).

The Hsp60 chaperonins

Chaperonins are divided into two groups. To the first group belong chaperonins that are mostly found in prokaryotes, mitochondria, and plastids and the second group comprises chaperonins from archaea and the eukaryotic cytosol (Gutsche et al, 1999; Spiess et al, 2004). A member of the second group is the cytosolic TRiC complex (TCP1 ring complex) which is located to eukaryotic cytosol (Gao et al, 1992; Yaffe et al, 1992). TRiC is a 1MDa hetero-oligomeric complex consisting of two identical stacked rings, each composed of eight paralogous subunits, surrounding a central cavity. It has been shown that TRiC plays an important role in the folding of various cellular proteins, including actin and tubulin (Gomez-Puertas et al, 2004; Sternlicht et al, 1993). A TRiC client protein is encapsulated within the central cavity enabling the folding of the substrate in an ATP-dependent manner. The folding process within the chamber is critically dependent on the closure of a built-in lid. The hydrolysis of ATP provides the energy required for the mechanistically closing of the chamber by the built-in lid (Douglas et al, 2011; McCallum et al, 2000; Meyer et al, 2003).

The Hsp100 chaperone family

The yeast chaperone Hsp104, a member of the Hsp100 chaperone family, is a further important component of the cytosolic protein quality control system. With the assistance of Hsp70s and Hsp40s, Hsp104 is able to resolve disordered protein aggregates, giving these proteins a new possibility to refold into their native structure (Glover & Lindquist, 1998; Winkler et al, 2012). Yeast Hsp104 contains two AAA+ nucleotide-binding domains (NBD1 and NBD2) that are separated by a distinctive coiled-coil middle domain (Doyle & Wickner, 2009). Upon binding of ATP to NBD2 of Hsp104 the chaperone switches to an active mode forming a ring-shaped Hsp104 homohexamer (Parsell et al, 1994; Schirmer et al, 2001). In this active form, ATP hydrolysis, which is mainly driven by the first AAA+ nucleotide-binding domain (NBD1), is coupled with the unfolding of the aggregated protein, delivered and presented to the central pore of the Hsp104 homohexamer by the Hsp70s and Hsp40s. Here, the unfolded polypeptide is translocated from the aggregated surface of the protein, across the central pore of the Hsp104 to solution, giving a further chance to refold into the correct conformation (Lum et al, 2008; Lum et al, 2004).

Small heat shock proteins (sHsp)

The seven chaperones Hsp12, Hsp26, Hsp42 and Hsp31-34 represent the class of the so-called small heat shock proteins (sHsp), which have the common feature to work ATP-independent. With the exception of Hsp12, sHsp are exclusively located to the cytosol and interact under stress conditions with unfolded or partially folded proteins to prevent their aggregation (Friedrich et al, 2004; Stromer et al, 2004). For this purpose, it has been shown that Hsp26 and Hsp42 form barrel-shaped oligomers. It is assumed that these oligomers function as a "storage depot" of protein aggregates. In the presence of Hsp70- and Hsp100 chaperones close to these "storage depot", aggregated proteins can be eventually released for refolding (Cashikar et al, 2005; Haslbeck et al, 2005). Recent data showed a complete different role for Hsp12 under stress conditions, including heat shock, oxidative stress and high osmolarity. During stress, Hsp12 converts from intrinsically unfolded protein, which is located to the cytosol, to a compact folded protein that is located mainly at the plasma membrane. There, it increases the stability of the plasma membrane (Welker et al, 2010). The functions of the small heat shock chaperones Hsp31-34 are largely unknown.

3.2 Degradation of cytosolic proteins by intracellular proteolytic pathways

An important feature of living cells is to maintain a proper cellular protein homeostasis under different environmental conditions. Regulated and fine-tuned protein synthesis and its counterpart, the selective (or unselective) degradation of proteins keep the right balance of the cellular proteome. Not any more needed proteins, including membrane receptors, transcription factors, enzymes or cyclins from the cell cycle as well as proteins that exhibit proteotoxicity, including terminally misfolded proteins, mislocalized proteins and single protein subunits (”orphan proteins”) that are not integrated in their respective protein complexes are degraded by intracellular proteolytic pathways. In eukaryotic cells there are two major locations for protein degradation: the lysosome (or vacuole in yeast) and the proteasome (see Figure 2). While the vacuole, a membrane-surrounded cell organelle, is responsible for the degradation of extracellular, membrane-associated and long-lived intracellular proteins (Davis et al, 1993; Raths et al, 1993; Wolf, 2004) the proteasome, a multicatalytic-multifunctional proteinase, degrades short-lived- as well as abnormally folded proteins (Wolf & Hilt, 2004). However, the borders of the two systems are fluent as the physiological state of a cell can influence whether a cytosolic protein is degraded by the vacuole or by the proteasome. A cytosolic protein can be degraded by the proteasome under vegetative growth conditions whereas under starvation conditions it is subjected for degradation by the vacuole (Egner et al, 1993). As well, when the capacity of the proteasome is saturated or in case of proteasome–inhibition, the lysosomal pathway becomes the major clearance route for cytosolic proteins that are usually destined for proteasomal degradation (Rideout et al, 2004; Trombetta & Parodi, 2003).

3.2.1 The lysosome (vacuole) – system

The lysosome in higher eukaryotes and the vacuole, its correspondent organelle in *S. cerevisiae*, is a single membrane-surrounded cell organelle containing a wide spectrum of different digestive enzymes including lipases, amylases, proteases, nucleases and phosphatases. These enzymes ensure the bulk degradation of macromolecules and entire organelles imported by autophagosomes or endosomes as well as bacteria delivered from phagosomes. Besides its function as bulk degradation machinery for macromolecules the vacuole serves as storage compartment for amino acids, small ions and polyphosphates (Jones, 1997). All resident enzymes in the vacuole have an

optimum catalytic activity between a pH of 4.8 and 6.0. As the pH in the cytosol is about 7.2 protons are transported in an ATP-dependent process by proton-pumps against a concentration gradient (Nelson et al, 2000). The bulk degradation of long-lived cytosolic proteins is initiated by autophagy (Yorimitsu & Klionsky, 2005), a process where proteins are incorporated in double-membrane vesicles, called autophagosomes and delivered to the vacuole where they are finally degraded by an arsenal of vacuolar endopeptidases and exopeptidases (Achstetter et al, 1984; Jones, 1991). The uptake of cytosolic proteins by autophagy coupled with their degradation in the vacuole becomes more and more important under starvation conditions. In that case, the total amount of cytosolic protein degradation in the vacuole increases from 40% to 85% to ensure the supply of amino acids for protein synthesis (Teichert et al, 1989). Although autophagy has long been considered as a non-specific process in eukaryotes it is now clear that the uptake process can also be specific. Cargo selection in the autophagy-related cytoplasm-to-vacuole trafficking (Cvt) pathway in yeast (Yorimitsu & Klionsky, 2005), e.g. mitophagy, ribophagy are specific autophagic pathways (Kirkin et al, 2009). A pathway distinct from autophagy is the chaperone-mediated autophagy (CMA) that has been found in mammals. The HSC70 chaperone recognizes cytosolic proteins, which contain a targeting motif (Lys-Phe-Glu-Arg-Gln) and delivers them to the lysosome (Dice, 2007; Massey et al, 2004; Yorimitsu & Klionsky, 2005).

3.2.2 The ubiquitin proteasome system

While extracellular, membrane-associated and long-lived intracellular proteins are clients for the lysosomal/vacuolar degradation machinery, short-lived proteins as well abnormally folded proteins are substrates of the ubiquitin proteasome system (Wolf & Hilt, 2004). Ubiquitin, which is present in the cytosol of all eukaryotes, is a small and highly conserved protein. Only 3 out of its 76 amino acids differ between yeast and human ubiquitin. One of the important functions of ubiquitin is to serve as a degradation marker for proteins. For this purpose ubiquitin is attached to a target protein (a process termed ubiquitination) by a three-step mechanism involving the sequential action of three different enzymes (Glickman & Ciechanover, 2002; Passmore & Barford, 2004). In the first step, the ubiquitin-activating enzyme (named E1) activates ubiquitin. At this, an E1 enzyme adenylates ubiquitin in which pyrophosphate is released from ATP. Then the C–terminal glycine residue of the ubiquitin-AMP-

intermediate forms a highly reactive thioester bond with a specific cysteine-residue of the E1 enzyme. In the second step the E1-bound ubiquitin is transferred to the active-site cysteine residue of an ubiquitin-conjugating enzyme (E2) where it is again connected via a thioester bond. In yeast only one gene encodes for an E1 enzyme (*UBA1*), whereas 13 genes have been described encoding E2 enzymes (*UBC1-UBC13*). The transfer of ubiquitin from the E2 to the target protein is the final step of the ubiquitination cascade carried out by an ubiquitin–protein ligase (E3 enzyme). In this process, ubiquitin is covalently attached via its C–terminal glycine residue with an internal lysine residue of the protein substrate, forming an isopeptide bond. E3s catalyze also the transfer of activated ubiquitin from an E2 to internal lysines present in ubiquitin, yielding a polyubiquitin chain anchored onto the protein substrate (more details about E3s, see chapter 3.3.2.1). As ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48 and K63) different linkages can be formed within a polyubiquitin chain. In addition, linear chains can be formed between the N- and C-termini of ubiquitin (Walczak et al, 2012; Wolf, 2011). Whereas less is known about the function of most of the ubiquitin linkages, it has been well documented that most of the proteins attached with a K48-linked polyubiquitin chain are degraded by the 26S proteasome. In contrast, ubiquitin chains formed through K63 attached to a substrate have been shown to play a role in DNA repair and endocytosis (Woelk et al, 2007). However, non-proteolytic functions for K48-linked- and proteolytic function for Lys63-linked polyubiquitin chains have been reported (Li & Ye, 2008). A fourth enzyme, the E4 (ubiquitin–elongase) functions in the elongation of already attached polyubiquitin chains onto a substrate (Koegl et al, 1999). The last step in the elimination process of polyubiquitinated proteins occurs in the proteasome, a large multi-subunit protease complex which is present in the cytosol and in the nucleus (Wolf & Hilt, 2004). A schematic illustration of the ubiquitin proteasome system is illustrated in Figure 2. The proteasome (26S) has a molecular mass of about 2.5 MDa and is the most common form of the proteasome. It is composed of a barrel-shaped 20S core particle containing the protease activity and two 19S regulatory particles mediating the recognition and binding of polyubiquitinated protein substrates. Furthermore, the 19S regulatory particle performs unfolding by consumption of ATP, deubiquitination with assistance of deubiquitinating enzymes (DUBs) and active transmission of the protein into the chamber of the 20S core (Wolf & Hilt, 2004).

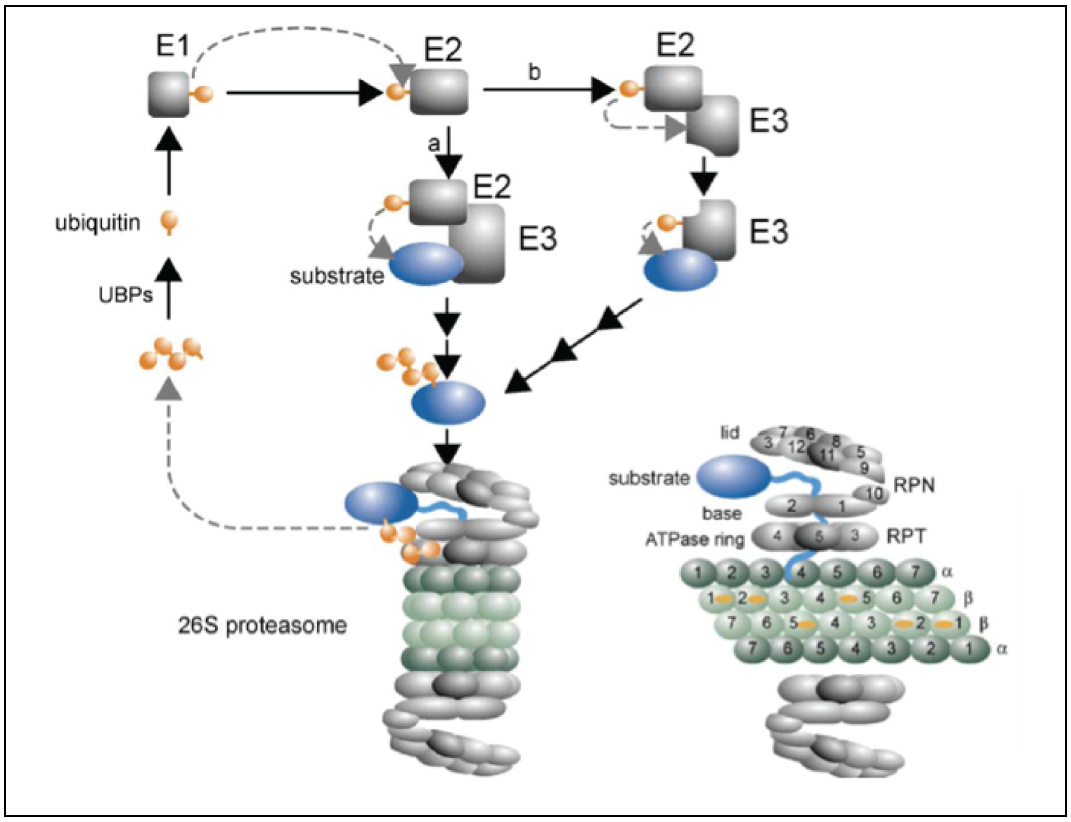

Figure 2 The ubiquitin proteasome system (reproduced from Wolf and Hilt, 2004)

In an ATP consuming step, ubiquitin is activated by binding via its carboxy terminus to a cysteine residue of the ubiquitin-activating enzyme (E1) resulting in a thiol-ester linkage. Then, the activated ubiquitin is transferred from the E1 to an ubiquitin-conjugating enzyme (E2). An ubiquitin-protein ligase (E3) containing a RING domain (a) binds the E2-ubiquitin via the E2 enzyme and transfers the ubiquitin directly from the E2 onto a lysine residue of the substrate. An E3 ligase containing an HECT domain (b) first binds and then transfers ubiquitin onto the substrate. The polyubiquitinated substrate is then recognized via the polyubiquitin chain by the 19S cap (grey subunits) of the 26S proteasome. The polyubiquitin chain is cleaved off by deubiquitinating enzymes and recycled to single ubiquitin molecules by ubiquitin-specific proteases (UBPs). The substrate gets unfolded and translocated into the 20S core chamber (green subunits) where it is digested into small oligopeptides.

The 20S core consists of four stacked hollow rings, each containing seven distinct but related subunits (Baumeister et al, 1988; Lasker et al, 2012), which are highly conserved among all eukaryotes. While the two outer rings with their seven α-subunits form the entrance for an unfolded, deubiquitinated polypeptide, the two inner rings provide the enzymatically activity digesting the substrate into small oligopeptides, varying between 3 and 23 amino acids in length (Heinemeyer et al, 1991; Kisselev et al, 1999;

Nussbaum et al, 1998). Three out of seven β-subunits of each inner ring posses proteolytic activity (Heinemeyer et al, 1997; Heinemeyer et al, 1991; Orlowski, 1990; Rivett, 1989). The β1-subunit hydrolyzes substrates after acidic or small hydrophobic amino acids (peptidyl-glutamyl-peptide cleaving activity), β2 cleaves after basic or small hydrophobic amino acids (trypsin-like cleaving activity) and the subunit β5 cuts the peptide bond after hydrophobic residues whether bulky or not (chymotrypsin-like cleaving activity) (Heinemeyer et al, 1997). After the cleavage of a protein destined for degradation by the proteasome, oligopeptides are released into the cytosol where they are further cut into amino acids by abundant cytosolic endopeptidases and amino-peptidases (Saric et al, 2004). The amino acids are available for the synthesis of new proteins or they can be guided to catabolic metabolism. In mammalian cells proteasomal peptide release is in addition required for triggering the immune response (Wang & Maldonado, 2006). Oligopeptides generated from proteasomal cleavage of foreign proteins also serve as antigenic peptides. Such peptides are presented at the plasma membrane surface and are then recognized by T-cells. A further role for proteasomes has been described in post-translational protein processing. An active subunit of the transcription factor NFκB, p50, is a product of proteasomal proteolysis from its inactive precursor form p105 (Palombella et al, 1994). More and more cellular processes have been found in which the proteasome plays a crucial and indispensable role. This is reflected in severe diseases, including cancer and neurological disorders that are associated with dysfunctional proteasomes (Hegde & Upadhya, 2007; Lecker et al, 2006; Lim & Tan, 2007).

3.2.2.1 Ubiquitin – protein – ligases (E3 enzymes)

Ubiquitin-protein-ligases (E3 enzymes) are responsible for the transfer of E2-conjugated ubiquitin molecules to the target protein and to an ubiquitin-(chain), which is already attached to a substrate. In addition to this function E3 ligases have also the ability to recognize very specifically substrates destined for regulated proteolysis by the proteasome (Glickman & Ciechanover, 2002). Thus, eukaryotes possess a large number of E3s, each responsible for a limited set of substrates. One example in which an E3 enzyme directly recognizes a substrate is described for the N-end rule pathway. The N–end rule pathway has been found in different organisms including *E. coli*, *S. cerevisiae* and mammalian cells (Varshavsky, 2011). When synthesized on ribosomes the

first amino acid at the N-terminus of a nascent polypeptide chain is methionine. According to the Sherman rule, this amino acid can be only cleaved when the side chain of the second N-terminal amino acid has a radius of gyration of less than 1.29 Å (Moerschell et al, 1990; Sherman et al, 1985). The new exposed N-terminal amino acid of a protein is either a stabilizing – or a destabilizing residue. The latter is directly recognized by the E3 RING ligase Ubr1. Then, the substrate is polyubiquitinated by Ubr1 and subsequently degraded by the 26S proteasome. The N-end rule component Ubc2 is the corresponding E2 enzyme of Ubr1. A destabilizing amino acid residue at the N–terminus of a protein together with an internal lysine (or lysines) represents a degradation signal, termed "N-degron". It determines the *in vivo* half-life of a protein (Bachmair et al, 1986; Varshavsky, 2011). In eukaryotic cells, it has been found that N-terminal amino acids, including Arg, Lys, Phe, Leu, Trp, Ile, His and Tyr are primary destabilizing residues. The N-terminal amino acids Asp and Glu represent secondary-, Asn and Gln tertiary destabilizing residues. N-terminal Asn or Gln are converted to Asp and Glu, respectively, catalyzed by the amidase Nta1. In turn, Asp and Glu are arginylated by the arginyl-transferase Ate1 resulting in the primary destabilizing amino acid residue Arg (Varshavsky, 2011). Stabilizing N-terminal amino acids are Val, Gly, Pro, Thr, Ser, Ala and Met. Further studies have shown that Ubr1 possess at least three substrate-binding sites (Turner et al, 2000; Xia et al, 2008). The type-1 substrate-binding site of Ubr1 recognizes specifically substrates with a basic N-terminal amino acid residue (Arg, Lys and His), whereas the type-2 substrate-binding site has a high affinity to substrates with a bulky hydrophobic or aromatic N-terminal amino acid residue (Phe, Leu, Trp, Tyr and Ile). The transcription repressor of the peptide transporter Ptr2, Cup9, binds via an internal degron to a third substrate-binding site of Ubr1. Finally, the substrate is polyubiquitinated by Ubr1 and then targeted to the 26S proteasome for degradation. Specific recognition of a certain protein substrate and transfer of ubiquitin molecules to that substrate are important functions of E3 ligases. The mode of ubiquitin transfer to the substrate occurs differently through the two known E3 ligase classes (Budhidarmo et al, 2012; Kee & Huibregtse, 2007; Metzger et al, 2012). In one case ubiquitin is transferred from the E2, which is bound to the E3, directly to the target protein (Figure 2 pathway a) (RING finger ligases, U–Box ligases). In the other case ubiquitin is transferred from the E2 to the E3, which is attached to the substrate (Figure 2, pathway b) (HECT ligases). Then the E3-attached ubiquitin is donated to the E3-bound substrate. RING–finger proteins have a characteristic RING–finger domain, an

spatial arranged octet composed of cysteine- and histidine residues that constitute a zinc ion–binding structure (Borden, 2000). Besides the "classical" RING finger structure, composed of eight cysteine- and histidine residues coordinating two zinc ions there are several proteins with RING finger like domains, exhibiting a different arrangement or even lacking some of these residues. The function of RING finger proteins is not restricted to protein degradation only. They are also involved in transcription, RNA transport, signal transduction, organelle transport and recombination (Saurin et al, 1996). However, the common function of all RING–finger proteins is to serve as a platform for protein-protein interactions. For substrate ubiquitination, the E2-conjugated ubiquitin binds to the RING domain of the RING E3, whereas the substrate interacts with another protein-protein interaction domain of the E3 RING ligase. Thus, both ubiquitin and substrate are brought into close proximity, resulting in ubiquitination of the substrate (Borden, 2000; Jackson et al, 2000; Joazeiro & Weissman, 2000). This mode of an indirect ubiquitin transfer ("indirect" confers to no binding of ubiquitin by the E3) is also a characteristic in a RING finger related class, the U-box E3 ligases. Here, the U–Box domain mediates binding of E2-ubiquitin and E3 ligase activity. This motif differs from the classical RING finger domain by lacking the hallmark metal-chelating residues (Aravind & Koonin, 2000). Well-known and described U-Box E3s are the yeast Ufd2 that possess E3 and E4 function (Koegl et al, 1999) as well as the human E3 CHIP, which exhibits chaperone-dependent E3 ligase activity (Ballinger et al, 1999; Connell et al, 2001). The RING finger domain-containing E3 family in eukaryotic cells includes also multimeric protein complexes that harbour, among others, a RING finger protein subunit mediating the binding of E2-conjugated ubiquitin. These protein complexes are implicated in the degradation of certain protein substrates. For instance, the Anaphase Promoting Complex (APC) is involved in degradation of cell cycle regulators whereas the Skp1-Cullin/Cdc53-F-box protein (SCF) RING finger complexes are engaged in degradation of signal- and cell cycle-induced phosphorylated proteins (Deshaies, 1999; Page & Hieter, 1999).

HECT–ligases (Homolog to E6-AP C–Terminus) represent the second major class of protein–ubiquitin ligases. In contrast to RING–ligases, HECT-E3s catalyze the final attachment of ubiquitin to the protein substrate through their C–terminal HECT-domain (Huibregtse et al, 1995; Scheffner et al, 1995; Schwarz et al, 1998). More precisely, a HECT-ligase binds an ubiquitin-conjugated E2 enzyme and transfers, in a still unknown catalytic mechanism, ubiquitin from the E2 cysteine to a conserved cysteine

residue within the HECT-domain of the E3, thereby forming an intermediate thioester bond. In the next step, HECT-E3 attaches the ubiquitin molecule to a lysine residue of the protein substrate. Based on structural studies for different HECT-E3s two models have been described so far for this final step of protein ubiquitination. The 'sequential addition' model proposes that an ubiquitin moiety is picked up from the E2 by a structurally element of HECT-E3 (termed C lobe), which rotates around its hinge loop and transfers the ubiquitin onto the substrate and adds additional ubiquitin molecules in a sequential manner. By contrast, the 'indexation' model suggests that the C lobe forms at first a tetra-ubiquitin chain on itself before transferring it onto the substrate (Passmore & Barford, 2004). The mechanisms, how RING E3- and HECT E3 ligases transfer ubiquitin molecules to a substrate are illustrated in Figure 2.

The association of ubiquitin protein ligases and several diseases, including Alzheimer, Parkinson, Amyotrophic lateral sclerosis, Huntington's disease (Ardley & Robinson, 2004) and many other neurodegenerative diseases emphasise their crucial role in the UPS. Due to these findings E3 ligases becoming very interesting targets for medical treatment. In contrast to the inhibition of the proteasome, targeting of specific E3's would selectively stabilize (or destabilize) a specific cellular protein regulated by this E3 ligase.

3.3 Diverse strategies and concepts of cytosolic protein quality control in yeast

In the past 15 years and beyond different approaches and different model substrates were used to study cytosolic protein quality control (CytoQC) in eukaryotic cells. Here, new components and new mechanisms have been elucidated making clear that cells have diverse strategies to manage the degradation of native or misfolded proteins located to the cytosol. Varshavsky and his colleagues discovered that the N-terminus of a protein can influence its *in vivo* half-life, named the N-end rule, in which the RING ligase Ubr1 is required for recognition and, together with the E2 Ubc2, for polyubiquitination of the corresponding substrate destined for proteasomal degradation (see also chapter 3.2.2.1). Our lab used ER import defective CPY variants (ΔssCPY, ΔssCPY*, ΔssCPY*-GFP, ΔssCPY*-Leu2-myc$_{13}$ and ΔssCPY*-GFP-cODC), which represent cytosolic misfolded proteins, to study CytoQC. Whereas the E3 ligase Ubr1 was identified to be necessary for the degradation of ΔssCPY*-Leu2-myc$_{13}$, the nuclear E3 ligase San1 is mainly involved in the proteasomal degradation of ΔssCPY, ΔssCPY* and ΔssCPY*-GFP (Eisele, 2011; Eisele & Wolf, 2008; Heck et al, 2010). In a previous study, it could be shown that the Hsp70 chaperone Ssa1 and the Hsp40 chaperone Ydj1 are necessary for the degradation of these cytosolic misfolded proteins (Park et al, 2007). Ssa1 and Ydj1 keep the substrate soluble and make it therefore accessible for the ubiquitin proteasome system. Whereas Ssa1 seems to play a general role for the removal of proteins destined for proteasomal degradation Ydj1 is partly involved (Heck et al, 2010; McClellan et al, 2005; Metzger et al, 2008; Nillegoda et al, 2010; Prasad et al, 2010). Folding of the von Hippel-Lindau (VHL) tumor-suppresor protein is coupled with its assembly into a ternary complex, consisting of VHL, ElonginB and ElonginC. In the absence of the binding partners ElonginB and ElonginC, proteasomal degradation of VHL requires, among others, the Hsp70 chaperone Ssa1, but surprisingly not the Hsp70 cochaperone Ydj1 (McClellan et al, 2005).

Different research groups have discovered a complete new strategy degrading cytosolic misfolded proteins. Ng and his colleagues used an ER-import defective Proteinase A protein (ΔssPrA) and GFP with an internal deletion disrupting the well-ordered β-barrel structure (Δ2GFP). Both model substrates are misfolded and localized to the cytosol. Surprisingly, they are imported into the nucleus to undergo ubiquitination by the nuclear E3 ligase San1 followed by proteasomal degradation. It

could be shown that the nuclear import of these cytosolic misfolded substrates is mediated by the Hsp70 chaperone Ssa1 (Prasad et al, 2010). Using ΔssCPY*-GFP as model substrate, it has been shown that its degradation is also mainly dependent on the E3 ligase San1 (Heck et al, 2010). The mechanism how San1 recognizes client substrates was proposed by Gardner and his colleagues. Intrinsically disordered regions, located at the N- and C-terminus of the nuclear ligase San1, are able to bind misfolded substrates (Rosenbaum et al, 2011). A completely different pathway to degrade cytosolic proteins was found by Metzger et al., 2008. Here, the CL1 degron, which consists of 16 amino acids forming an amphipathic helix, was C-terminally fused to the orotidine-5'-phosphate (OMP) decarboxylase (Ura3-CL1), making the otherwise stable Ura3 protein proteolytically instable. Unexpectedly, the proteasomal degradation of this artificial cytosolic substrate was dependent on components of the ERAD system. The two E2 conjugating enzymes Ubc6 and Ubc7 as well as the E3 ligase Doa10 are involved in the ubiquitination process of Ura3-CL1 (Gilon et al, 1998; Gilon et al, 2000; Metzger et al, 2008). Subsequently further components were identified, including Ssa1, Ydj1 and the Cdc48-Npl4-Ufd1 complex, which contribute to the removal of this degron-containing substrate (Metzger et al, 2008). It was therefore proposed that the cytosolic face of the endoplasmic reticulum might serve as a "platform" for degradation of certain cytosolic proteins.

Further studies were performed supporting that the E3 ligase Ubr1 is not only restricted to act in the N-end rule pathway. Nillegoda et al. 2010, have shown that upon Hsp90 inhibition by the competitive inhibitor geldanamycin the degradation of newly synthesized protein kinase Tpk2, which is a client of Hsp90, is dependent on Ubr1. Furthermore they showed an involvement of Ubr1 in the degradation of heat-denatured luciferase. A recent study, done by Farzin-Khosrow-Khavar in 2012, supports this role in degradation of heat-denatured proteins. By using 22 different thermosensitive protein mutants from *S. cerevisiae*, it was figured out that certain thermosensitive substrates at non-permissive temperature were completely stabilized in a *UBR1* deletion strain whereas some were only fully stable in a double deletion strain of *UBR1* and *SAN1* (Khosrow-Khavar et al, 2012). All these studies show that the cytosolic ligase Ubr1 and the nuclear ligase San1 play a dominant role in degradation of cytosolic misfolded proteins.

Beside soluble proteins destined for degradation, cells have evolved strategies to deal with insoluble proteins and protein aggregates. As such aggregates are associated with

some neurodegenerative diseases, including Parkinson's and Alzheimer disease as well as Huntington disease it is of high interest to understand the mechanisms how protein aggregates arise and how the cell manages this problem to avoid cell damage or cell death caused by these protein aggregates (Caughey & Lansbury, 2003; Chiti & Dobson, 2006). Different kinds of stress, like heat stress, oxidative stress, chemical modification or exposure to chemical agents increase the amount of misfolded proteins provoking the formation of protein aggregates. When the degradation capacity of these misfolded proteins is exhausted the generation of protein aggregates is a logical consequence. Kaganovich et al., 2008, discovered a new strategy how eukaryotic cells manage these aggregates in order to prevent cell damage. It has been found in yeast and in mammalian cells that they possess two intracellular compartments where they store aggregation prone proteins (Kaganovich et al, 2008). In the so-called juxtanuclear quality control compartment (JUNQ), which contains proteasomes and is formed upon the induction of cellular stress, soluble ubiquitinated misfolded proteins are accumulated. In contrast, non-ubiquitinated and terminally aggregated proteins are located within the so-called insoluble protein deposit (IPOD). Furthermore, the IPOD is free of proteasomes and can be also be found in unstressed cells. Another approach to dispose aggregated proteins is described by Liu et al., 2010. They observed an active retrograde transport of protein aggregates from the daughter to the mother cell during cell division. Here, the Hsp104-containing protein aggregates are attached to an actin cable, which is synthesized continuously by the TRiC chaperonin, guided back to the mother cell (Liu et al, 2010).

3.4 Objective of the thesis

The main components responsible for protein quality control in eukaryotic cells are the molecular chaperones and the ubiquitin proteasome system. In contrast to the well-described protein quality control and degradation system in the endoplasmic reticulum the mechanisms how eukaryotic cells cope with misfolded proteins in other cell compartments (e.g. nucleus, mitochondria or in the cytoplasm) are still poorly understood. This study focuses on the cytosolic protein quality control and the degradation of a naturally unassembled protein subunit, named orphan Fas2. One can assume that the synthesis of subunits of a multimeric protein complex is never equal unless one and the same promoter controls the synthesis. The excess of a subunit must most likely be removed from the cell in order to avoid unspecific interactions with other proteins or substrates, which would be unproductive or even harmful for the cell. Egner et al., (1993) reported that the Fas2 subunit, when assembled into the fatty acid synthase complex, is a long lived protein, while an orphan Fas2 becomes a short lived protein, which is exclusively degraded by the 26S proteasome. The goal of this study was to identify new components involved in the quality control process of orphan Fas2 and particularly to understand how these new factors function in the degradation process of a naturally occurring orphan protein. For this purpose the experimental setup was based on chromosomal expression of Fas2 under its native promoter, in the absence of its partner protein Fas1, making Fas2 an orphan protein (Figure 3). Using a *FAS1* deletion strain, the degradation kinetics of orphan Fas2 were investigated in the presence of gene deletion(s) or –mutations, coding for chaperones and components of the ubiquitin proteasome system as well as other factors. Additionally, protein-protein interaction databases were screened to find candidates that could possibly be involved in orphan Fas2 degradation. All proteins associated with Fas2 or components involved its degradation process, were further examined. Thus, this thesis contributes to the understanding how a natural, non-mutated orphan protein in the cytosol is recognized and degraded by the cytosolic protein quality control system in yeast.

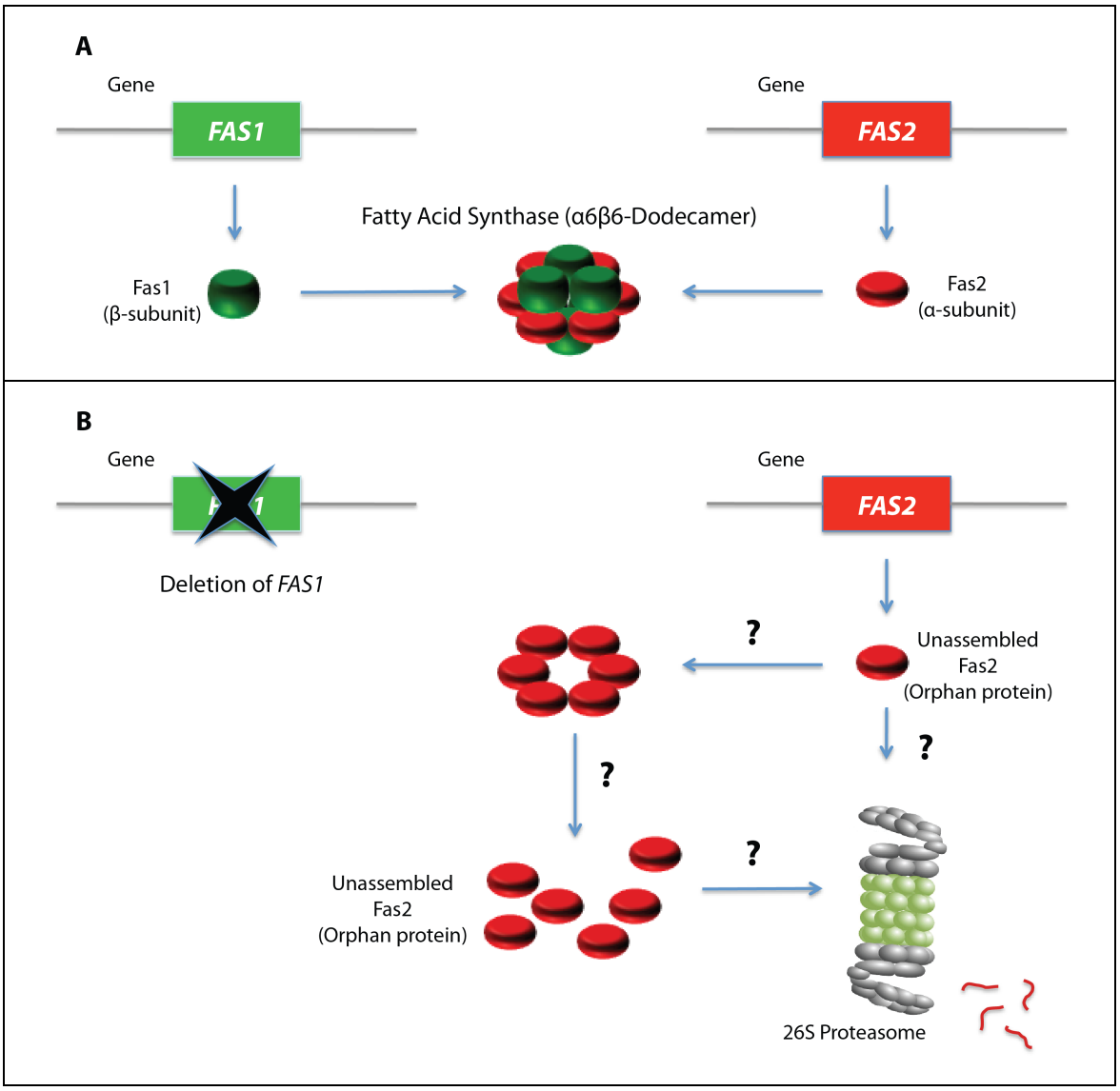

Figure 3. The experimental design to investigate the proteolytical fate of the unassembled protein subunit Fas2. A. The fatty acid synthase (FAS) genes, *FAS1* and *FAS2*, encode the β-subunit (Fas1) and the α-subunit (Fas2), respectively. With a molecular weight of 2.6 MDa, the yeast FAS-complex consists of six copies each of Fas2 (M_r = 207 KDa) and Fas1 (M_r = 229 KDa) subunits forming a large barrel-shaped dodecamer. The six Fas2 subunits are organized in a wheel-like structure covered by three Fas1 subunits each on top and bottom of the wheel. **B.** In order to follow the proteasome-mediated degradation of the unassembled Fas2 subunit (orphan Fas2) its complex partner Fas1 was removed by the deletion of *FAS1* in a wild type strain. The goal of this work was to identify new components that are required for the proteasome-mediated elimination of orphan Fas2. In this context it was important to determine whether orphan Fas2 exists as monomer or it is organized as FAS assembly intermediate(s).

4 Materials and methods

4.1 Material and equipment

4.1.1 Strains of *Saccharomyces cerevisiae*

Strain	Genotype	Source
YWO 0357 (W303-1A)	*Mata, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Chiang and Schekman, 1991
YWO 0365 (YPH499Y)	*Mata, ura3-52 his3-Δ200 leu2-Δ1 trp1Δ63 lys2-801 ade2-101 prc1-1*	Hiller et al., 1996
YWO 0511	W303-1B *Δpdr5::TRP1 prc1-1*	R. Plemper, 1998
YWO 0631	W303-1B *ydj1-2::HIS3 LEU2::ydj1-151 prc1-1*	P. Deak, 2001
YWO 0679	W303-1A *Δhsp104::KanMX prc1-1*	R. Hitt, 2003
YWO 0903 (W303-1B)	*Matalpha, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Chiang and Schekman, 1991
YWO 0907	W303-1B *Δubc8::KanMX*	T. Schüle
YWO 1161	W303-1B *hsp26::LEU2*	Susek and Lindquist, 1989
YWO 1163	W303-1B *hsp26::LEU2 hsp42::HygB*	Cashikar et al., 2005
YWO 1221	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
YWO 1234 (YCM762)	*Mata, cim3-1 ura3-52 leu2Δ1 his3Δ-200*	Gishlain et al., 1993
YWO 1435	W303-1A *Δubr1::loxP prc1-1*	F. Eisele, 2011
YWO 1443	W303-1B *cdc48-ts (T413R)*	F. Eisele, 2011
YWO 1590	W303-1A *Δssa2::loxP Δssa3(1126bp)::HIS5 Δssa4::loxP Δprc1::LEU2*	S. Besser, 2011
YWO 1591	W303-1A *ssa1-45 Δssa2::loxP Δssa3(1126bp)::HIS5 Δssa4::loxP Δprc1::LEU2*	S.Besser, 2011
YWO 1629	W303-1B *Δsan1::KanMX*	L. Barbin, 2009
YWO 1630	W303-1B *Δdoa10::KanMX*	L. Barbin, 2009
YWO 1750	W303-1A *prc1::loxP Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δubr1::HIS5*	F. Eisele, 2011
YWO 1751	W303-1A *ssa1-45 prc1::loxP Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δubr1::HIS5*	F. Eisele, 2011
YWO 1764	*Mata, his3Δ1::HIS3-sse1-CT3 leu2Δ0 met15Δ0 ura3Δ0 sse1Δ100::hphMX4 sse2Δ::KanMX*	C. Andréasson and B. Bukau
YWO 1765	*Mata, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sse1Δ100::hphMX4 sse2Δ::KanMX [p2H-PGPD-SNL1ΔTM]*	C. Andréasson and B. Bukau
YIA 3	W303-1A *Δubc2::KanMX prc1-1*	I. Amm, 2011
YMS 5	W303-1B *Δfas1::HIS5*	This study
YMS 7	*Mata, cim3-1 ura3-52 leu2Δ1 his3Δ-200 Δfas1::HIS5*	This study
YMS 23	W303-1A *Δubr1::loxP prc1-1 Δfas1::HIS5*	This study
YMS 35	W303-1A *Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δprc1::LEU2 Δfas1::HIS5*	This study
YMS 36	W303-1A *ssa1-45 Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δprc1::LEU2 Δfas1::HIS5*	This study

YMS 42	W303-1B *hsp26::LEU2 Δfas1::HIS5*	This study
YMS 43	W303-1B *hsp26::LEU2 hsp42::HygB Δfas1::HIS5*	This study
YMS 45	W303-1B *cdc48-ts (T413R) Δfas1::HIS5*	This study
YMS 50	*MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 Δfas1::HIS5*	This study
YMS 51	W303-1B *FAS2-TAP-TRP1 Δfas1::HIS5*	This study
YMS 60	W303-1B *FAS2-TAP-TRP1*	This study
YMS 63	*Mata, his3Δ1::HIS3-sse1-CT3 leu2Δ0 met15Δ0 ura3Δ0 sse1Δ100::hphMX4 sse2Δ::KanMX Δfas1::URA3*	This study
YMS 64	W303-1B *FAS2-TAP-TRP1Δubr1::loxP prc1-1 Δfas1::HIS5*	This study
YMS 68	W303-1B *Δpep4::loxP Δprb1::loxP Δfas1::HIS5*	This study
YMS 73	W303-1B *Δdoa10::loxP Δfas1::HIS5*	This study
YMS 78	W303-1B *Δsan1::loxP Δfas1::HIS5*	This study
YMS 79	*Mata, his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sse1Δ100::hphMX4 sse2Δ::KanMX [p2H-PGPD-SNL1ΔTM] Δfas1::URA3*	This study
YMS 87	W303-1B *Δpdr5::TRP1 prc1-1 Δfas1::HIS5*	This study
YMS 91	W303-1B *Δsti1::HIS5 Δfas1::URA3*	This study
YMS 114	W303-1B *Δapj1::loxP Δfas1::HIS5*	This study
YMS 119	W303-1A *Δubc2::KanMX prc1-1 Δfas1::HIS5*	This study
YMS 121	W303-1B *Δubc8::KanMX Δfas1::HIS5*	This study
YMS 131	W303-1B *FAS2-EGFP-KanMX Δfas1::HIS5*	This study
YMS 132	W303-1A *FAS2-EGFP-KanMX Δubr1::loxP prc1-1 Δfas1::HIS5*	This study
YMS 146	W303-1A *ssa1-45 prc1::loxP Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δubr1::HIS5 Δfas1::URA3 FAS2-TAP-TRP1*	This study
YMS 152	W303-1B *Δxdj1::URA3 Δfas1::HIS5*	This study
YMS 153	W303-1A *prc1::loxP Δssa2::loxP Δssa3(1126bp)::loxP Δssa4::loxP Δubr1::HIS5 Δfas1::URA3 FAS2-TAP-TRP1*	This study
YMS 154	W303-1A *Δubc2::KanMX prc1-1 Δfas1::HIS5 Δubc4::URA3*	This study
YMS 155	W303-1B *Δubc4::URA3 Δfas1::HIS5*	This study
YMS 158	W303-1B *ydj1-2::HIS3 LEU2::ydj1-151 prc1-1 Δfas1::URA3*	This study
YMS 176	W303-1B *Δhul5::URA3 Δfas1::HIS5*	This study
YMS 180	W303-1B *FAS2-EGFP-KanMX*	This study
YMS 185	W303-1A *Δhsp104::KanMX Δfas1::HIS5 PRC1*	This study
YMS 186	*Mata, ura3-52 his3-Δ200 leu2-Δ1 trp1Δ63 lys2-801 ade2-101 prc1-1 Δfas1::HIS5*	This study

Table 1: Strains of *S.cerevisiae*

4.1.2 Strains of *Escherichia coli*

Strain	Genotype	Reference
DH5α	F-, endA1, hsdR17 (rk-, mk+), supE44, thi-1, recA1, gyrA96, relA1, s80ΔlacZM15	(Hanahan, 1983)
XL-10 Gold	endA1 glnV44 recA1 thi-1 gyrA96 relA1 lac Hte Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 tet^R F'[proAB $lacI^qZ\Delta M15$ Tn10(Tet^R Amy Cm^R)]	Stratagene

Table 2: Strains of *E. coli*

4.1.3 Plasmids

Plasmid name	Characteristics	Source
pUG6	*loxP-KanMX-loxP, Amp^R*	Gueldener et al., 1996
pUG27	*loxP-HIS5$^+$-loxP, Amp^R*	Gueldener et al., 2002
pUG72	*loxP-URA3-loxP, Amp^R*	Gueldener et al., 2002
pUG73	*loxP-LEU2-loxP, Amp^R*	Gueldener et al., 2002
pSH47	*P_{GAL1}::Cre, Amp^R, TRP1*	Gueldener et al., 1996
pYM12	Plasmid for EGFP-tagging of proteins at the C-terminus	Knop et al., 1999
pBS1479	Plasmid for TAP-tagging of proteins at the C-terminus, marker TRP1 from *K.lactis*	Rigaut, G, 1999
YEplac181	High copy LEU2-marked vector with *ADH1* promoter and *CYC1* terminator	Xia et al., 2008
pRB208	Ubr1-HA in YEplac181	Xia et al., 2008
pRB208IMI	Ubr1-HA (D176E) in YEplac181	Xia et al., 2008
pRB208IIMI	Ubr1-HA (P406S) in YEplac181	Xia et al., 2008
pFlagUBR1SBX	Flag-Ubr1 in YEplac181	Xia et al., 2008
pFlagUBR1MR1	Flag-Ubr1 (C1220S) in YEplac181	Xie and Varshavsky, 1999
Derivative of pSK146 (modified by I.Amm)	San1 (w/o NLS)-V5-HIS in pTS210	I. Amm, 2011 Prasad et al., 2010
pTS210	*P_{GAL1}; URA3, YCP*50	Prasad et al., 2010

Table 3: Plasmids

4.1.4 Oligonucleotides

All Oligonucleotides are given from 5′ to 3′ direction. Sequences that are homologous to DNA template are underscored.

GDFAS1FW	AGTATATTTATTCGCCACACCTAACTGCTCTATTATTCGCTCATT CAGCTGAAGCTTCGTACGC
GDFAS1REV	TTTCAAAGTTAAATATTTCTTACGGTTATATAATCACTTAAGAAA GCATAGGCCACTAGTGGATCTG
CPfas1CTRev	TGCAGAGATGTGCGGAATAG
CPFAS1FW	AGTCGCTGCATCATTCTCTC
GDPRA1FW	CTAGTATTTAATCCAAATAAAATTCAAACAAAAACCAAAACTAAC CAGCTGAAGCTTCGTACGC
GDPRA1REV	TAGATGGCAGAAAAGGATAGGGCGGAGAAGTAAGAAAAGTTTAGC GCATAGGCCACTAGTGGATCTG
CPpra1CTRev	TTCGCTTCTGCTTACATTGC
CPPRA1Rev	TATTGGGCCATACGATTACACG

GDPRB1FW	ATCGCCAATAAAAAAACAAACTAAACCTAATTCTAACAAGCAAAG CAGCTGAAGCTTCGTACGC
GDPRB1REV	AGAAAAAGAAAAAAAAAAGCAGCTGAAATTTTTCTAAATGAAGAA GCATAGGCCACTAGTGGATCTG
CPprb1CTRev	GCATAGGCCACTAGTGGATCTG
CPPRB1Rev	ACAACGGTGGTGGTCAAG
GDSTI1FW	GCTCCCAAATTCCTCACTGTAGCTACTAAAACAACCTATACGCAAGAAAG CAGCTGAAGCTTCGTACGC
GDSTI1REV	AAGCAGTAAAAAAAGAATTCAAGATAATAAAGTTATATTTCGTATTATTT GCATAGGCCACTAGTGGATCTG
CPsti1CTRev	ATGGCAAGCACTTCTTCAGG
CPSTI1FW	TCCCTGAAGCTATCGCAGATTG
GDXDJ1FW	CGCTTGGACCCAAAAGAAAGGGAAAAGTAAGTACGCAGGTGTAGTTTTGA CAGCTGAAGCTTCGTACGC
GDXDJ1REV	GATAACTTATGAAAAAAAAAAAAAAATAGAATAAAAAGTTATTGATGCCA GCATAGGCCACTAGTGGATCTG
CPxdj1CTRev	AGTGTTATCCAATCCGCCTTC
CPXDJ1FW	AACCTGGCAGAGTAGTAAGAC
GDAPJ1FW	AAACACATTTTTCTTTTGCTACCAGTGGACACAAGATAACGGAGA CAGCTGAAGCTTCGTACGC
GDAPJ1REV	ATAAAAATAGAAAATGTATAACATTACTGCATGACTATATAACCT GCATAGGCCACTAGTGGATCTG
CPapj1CTRev	AAAGATCCCTACTGCTGAAACC
CPAPJ1FW	GAAGTTATTGAAGACGACGACC
GDUBC4FW	TAAATTTCACTGACTATAGAGTACATACATAAACAAGCATCCAAAAAAAC CAGCTGAAGCTTCGTACGC
GDUBC4REV	AATCCCATATAAATCTTGCTTCTCTTTTTCAGCTGAGTAAGGACTTCTGT GCATAGGCCACTAGTGGATCTG
CPubc4CTRev	ACAGAGAACCAGTCATTGATCG
CPUBC4NTFW	ATCAAATGGCCGAGCAACAG
GDHUL5FW	AAAGCGTTATAATTTATCTCAAGCAGCATTTCAAAGGATTCTGACTTAAA CAGCTGAAGCTTCGTACGC
GDHUL5REV	AGAACTAGAAATCTGCCTATTAATTAATTACCTCCCTAATCTTCTGGGAA GCATAGGCCACTAGTGGATCTG
CPhul5CTRev	TTATGAGCCGGAATGTCCAG
CPHUL5FW	CTTCAAACCAGTTTCGGCATTC
GDUFD4FW	CTCAGGTGTGAAAATTTTGGATAAACATTTAAAATAGAAGAGTAGCCAAGC CAGCTGAAGCTTCGTACGC
GDUFD4REV	GAAATAATTTTGAAGTCATATAAAACTTAAATAGAAAAAGTAACTAAAAT GCATAGGCCACTAGTGGATCTG
CPufd4CTRev	TAGTTCTGGTACGTTGCCTTTC
CPUFD4FW	GAACGCTGAACATGGCTATAC
HIS5-FW	CGCCTCGACATCATCTGCCC
URA3-FW	TTACCACCATCCAATGCAGAC
KanB	GGATGTATGGGCTAAATG
KanC	CCTCGACATCATCTGCCC
HIS5 Probe FW	TTTGGACAAAGCTCCCTTACCTG
HIS5 Probe Rev	AACACTCCCTTCGTGCTTGG
URA3 Probe FW	TCACCTTCAACCTTAGGATCTC

URA3 Probe Rev	TAGAGCTGAGACTCATGCAAG
KanMX Probe FW	GATAATGTCGGGCAATCAGGTG
KanMX Probe Rev	ATTCCGACTCGTCCAACATC
FAS2-TAP-FW	CACGATGACCTCCAAGCTGTCGCGGTCGCCGTTTCTACTAAGAAA TCCATGGAAAAGAGAAG
FAS2-TAP-Rev	GAAGCGACACGTTACATATTAAAAGAGGGACTACGTAGTGCTCTCT TACGACTCACTATAGGG
FAS2-EGFP-FW	CACGATGACCTCCAAGCTGTCGCGGTCGCCGTTTCTACTAAGAAA CAGCTGAAGCTTCGTACGC
FAS2-EGFP-Rev	AAGCGACACGTTACATATTAAAAGAGGGACTACGTAGTGCTCTCT GCATAGGCCACTAGTGGATCTG

Table 4: Oligonucleotides

4.1.5 Antibodies

Antibody	Dilution	Source
Rabbit anti-FAS	1:10.000	Egner et al., 1993
Mouse anti-PGK	1:10.000	Molecular Probes
Mouse anti-HA, monoclonal, clone 16B12	1:10.000	Covance
Mouse anti-Flag, monoclonal, clone M2	1:10.000	Sigma Aldrich
Mouse anti-rabbit, HRPO-conjugated, monoclonal, Clone RG-96	1:10.000	Sigma Aldrich
HRP conjugated goat anti-mouse	1:10.000	Jackson Immuno Research
Rabbit anti-TAP, polyclonal	1:10.000	Open Biosystems
Rabbit anti-GFP	1:5.000	Invitrogen
rabbit anti-G6PDH	1:10.000	Sigma Aldrich
Rabbit anti-Cdc48	1:10.000	T. Sommer

Table 5: Antibodies

4.1.6 Chemicals, kits and enzymes

Enzymes:

RNase	peqlab
Restriction enzymes	Fermentas
Velocity DNA-Polymerase	Bioline
Taq–DNA–Polymerase	Genaxxon Bioscience
Acetylated Trypsin (V-S type)	Sigma-Aldrich

Kits:

Mini Prep	GeneJET™ Plasmid Miniprep Kit, Fermentas
	peqGOLD Plasmid Miniprep Kit I
Immunodetection	ECL Western Blotting Substrate, Pierce
Gel extraction	Machery Nagel

Chemicals: were provided by Roth, Sigma-Aldrich, Fluka and Fisher Scientific

4.2 Cultivation of *S. cerevisiae* and *E. coli*

For cultivation of *S. cerevisiae* and *E. coli* standard media were used (Sambrook et al., 1989; Guthrie and Fink, 1991; Ausubel et al., 1992). Yeast strains lacking *FAS1* or / and *FAS2* were grown in media supplemented with fatty acid.

4.2.1 Media and cultivation conditions of *S. cerevisiae*

When not otherwise indicated: Percentage values (w/v)

YPD–media (pH 5.5)	1 %	Yeast extract
	2 %	Bacto peptone
	2 %	D-Glucose
YPD-FA-media	1 %	Yeast extract
	2 %	Bacto peptone
	2 %	D-Glucose
	0.03%	Myristic acid
	1% (v/v)	Tween 40
CM–media (pH 5.6)	0,67 %	Yeast nitrogen base w/o amino acids
	2 %	D-Glucose
	0.0117%	L-Alanin, L-Arginine, L-Aspargine, L-Aspartat, L-Cysteine, L-Glutamine, L-Glycine, L-Isoleucine, L-Methionine, L-Phenylalanine, L-Proline, L-Serine, L-Threonine, L-Tyrosine, L-Valine, myo-Inosit
	0.00117%	p-Aminobenzoeacid
YCM-FA-media	CM-media	
	0.03%	Myristic acid
	1% (v/v)	Tween40
	0.05%	Yeast extract

YPG-media	1 %	Yeast extract
	2 %	Bacto peptone
	2 %	Galactose (sterile filtrated)
YPG-FA-media	YPG-media	
	0.03%	Myristic acid
	1% (v/v)	Tween 40

Petri dishes contained additional 2 % Bacto® agar and for all media double distilled water (ddH_2O) was used. For strain selection containing the *KanMX* gene YPD-media were supplemented with geneticin sulfate (G418) at a final concentration of 300 mg/l.
S. cerevisiae was grown either in liquid media in an erlenmeyer flask on a shaker (150 rpm) or on solid media in a petri dish. On solid media cells were either streaked with a sterile toothpick or, as cell suspension, plated with a Trigalski applicator. Disposable sterile plastic sticks were used to inoculate cell culture in liquid media. Cells were grown at 30°C. Temperature sensitive mutants were grown either at 25°C or at 37°C. Growth state of a liquid cell culture was monitored by measuring the optical density at 600 nm wavelength from an 1:10 or 1.50 dilution (in respective media). One OD_{600} means about $2 * 10^7$ cells per ml. For long term storage fresh grown yeast cells (from one single colony picked and on fresh solid media streaked) were mixed in sterile 15% (v/v) glycerol and stored at – 80°C.

4.2.2 Media and cultivation conditions of *E. coli*

LB–media	1 %	Bacto tryptone
	0.5 %	Bacto yeast extract
	0.5 %	NaCl
SOC-media	2 %	Bacto tryptone
	0.5 %	Yeast extract
	0.4 %	D-Glucose
	10 mM	NaCl
	10 mM	$MgCl_2$
	10 mM	$MgSO_4$
	2.5 mM	KCl

For plasmid selection media were supplemented with ampicillin at a final concentration of 100 mg/l. Petri dishes contained additional 2 % Bacto® agar and for all media double distilled water (ddH_2O) was used.
E. coli strains were grown either in liquid media in an Erlenmeyer flask or glass tube on a shaker (220 rpm), or on solid media on a petri dish. LB–media was used as rich media

and the growth temperature was 37°C. In case for selection of ampicillin-resistant strains ampicillin was added to a final concentration of 100 mg/l. Growth state of a liquid cell culture was monitored by measuring the optical density at 600 nm wavelength from a 1:10 dilution (in respective media). For long term storage one volume (e.g. 300 µl) of fresh grown cells from a liquid culture was mixed with one volume of 60% sterile glycerol and stored at – 80°C.

4.3 Molecular – and cell biology methods

4.3.1 Generation and transformation of heat-competent *E. coli* cells

Generation and transformation of DH5α heat competent cells were performed according to the Inoue method (Molecular Cloning – A laboratory manual, 3rd edition, Sambrook and Russell). The final transformation efficiency was about 1 * 10^8 colony forming units / µg plasmid DNA.

4.3.2 Isolation of plasmid DNA from *E. coli*

Plasmids were isolated with the peqGOLD plasmid purification kit I from Peqlab or with GeneJET® Plasmid minipreparation kit from Fermentas according to manual instructions.

4.3.3 Isolation of chromosomal yeast DNA

5 ml of an YPD-overnight-culture (16–24h) was harvested by centrifugation at 3000 rpm for 4 min. The supernatant was discarded and remaining media was removed by washing with 1 ml ddH_2O, performed in a 1.5 ml safe lock eppendorf tube. In order to lyse the cells and to extract the chromosomal DNA, 200 µl of breaking buffer, 200 µl of phenol-chloroform-isoamylalcohol (under the hood) and 300 µl of glass beads were added to the cell pellet followed by 10 min vortexing at 4°C. Upon addition of 200 µl ddH_2O the sample was spun down at maximum speed (13.000 rpm) for 10 min. The upper aqueous phase (approximately 300 µl), which contains the chromosomal DNA, was transferred into a new 1.5 ml eppendorf tube. In order to precipitate the DNA 1 ml of 100% ice-cold ethanol was added, carefully mixed by inverting the tube 4 to 6 times (this step is important to avoid the formation of water crystals that can break the DNA) and incubated at –80°C for at least 20 min. Then, the sample was centrifuged at maximum speed for 10 min at 4°C and the s/n was aspirated by a vacuum pump. To digest RNA the DNA pellet was incubated with 400 µl ddH_2O and 3 µl RNase (10 µg/µl) at

37°C for 10 min. To precipitate the DNA again 10 µl of 5M NH_4Ac + 1 ml of 100% ice-cold ethanol were added and the sample was incubated at
–80°C for 15 min. After a further centrifugation step at maximum speed for 10 min at 4°C the DNA was washed with 1 ml of 70% ethanol, dried for 5 minutes under the hood and dissolved in 50 µl of ddH_20 by incubating at 37°C for 10 min. When not further processed, DNA was stored at 4°C.

Breaking buffer:	100 mM	NaCl
	1%	SDS
	10 mM	Tris-HCl (pH 8.0)
	1 mM	EDTA (pH 8.0)
	2% (v/v)	Triton X100

4.3.4 Digestion of plasmid DNA by restriction endonucleases

In order to validate the correct isolated plasmid DNA, it was digested by selected restriction endonucleases in total volume of 20 µl for 2h at 37°C. The digested DNA fragments were separated and analyzed by agarose gel electrophoresis.

4.3.5 Agarose gel electrophoresis

The separation and analysis of DNA fragments was performed by agarose gel electrophoresis. Here, the DNA (plasmids or chromosomal DNA) was mixed with DNA sample buffer, loaded onto a 0.8% agarose gel containing ethidium bromide (0.5 µg/ml) and separated at a voltage of 120 volts for about 30 min. The run of the gel was performed in TAE buffer. In order to determine the size of the separated DNA fragments 4µl of 1Kb DNA ladder was loaded onto the agarose gel. Gel pictures were taken under UV-light (302 nm) by the MWG Biotech documentation system.

TAE buffer:	40 mM	Tris/Acetic acid pH 7.0
	1 mM	EDTA pH 8.0
DNA-sample buffer:	50% (v/v)	Glycerol in 10x TAE buffer
	0.1% (w/v)	Bromphenolblue

4.3.6 Polymerase chain reaction

Polymerase chain reaction was used for several applications.

a) Generation of gene deletion cassettes
b) Generation of cassettes for C-terminal epitope-tagging of chromosomal yeast genes
c) Generation of DNA probes for Southern blot
d) Verification of specific genes or gene deletion on chromosomal yeast DNA (Colony-PCR)

In general, the total volume of a PCR mix was 50 µl (and 25 µl for colony PCR) and prepared in appropriate PCR-eppendorf tubes as described in Table 6. The amplification of the DNA template in the PCR mix was performed in a thermocycler from Biometra™. The chosen annealing temperature for the primers depends on their GC content and was determined by ApE software. The PCR reaction conditions in the thermocycler are shown in Table 7. In order to verify the PCR product 2 µl of the reaction mix was diluted in 16 µl ddH_20 + 2 µl 10x DNA sample buffer and analyzed onto a 0.8% agarose gel.

Reagent	Volume	Final concentration/activity
ddH_20	36-x µl	
5x HF buffer	10 µl	1x
dNTPs (10 µM each)	1 µl	200 nM (each)
Template DNA	x µl	Plasmid: 0.2 ng/ µl chromosomal DNA: 50 ng / µl
Forward primer (10 µM)	1 µl	200 nM
Reverse primer (10 µM)	1 µl	200 nM
DNA-Polymerase	1 µl	2U/µl

Table 6: Reagents and volumes used in a PCR reaction mix

Step	Temperature [°C]	Time
1. Preheating	98	Pause mode (∞)
2. Denaturing	98	2 min
3. Denaturing	98	30 sec
4. Annealing	50-52	30 sec
5. Elongation	72	1min/kb for Taq; 15s/kb for Velocity
6. Final elongation	72	4 min
7. End	4	Pause mode (∞)

Table 7: Program for DNA amplification in a PCR reaction

Colony PCR was applied for verification of a gene deletion in yeast. Little amount of fresh grown yeast cells on solid media were picked with a yellow tip, swirled into 30 µl of 0.02 M NaOH and incubated at 95°C for 10 min in a thermoshaker. After cell debris was spun down (5000 rpm, 1 min) 2 µl of the s/n was used as template in a 25 µl PCR reaction.

4.3.7 Yeast transformation

For plasmid- or PCR product transformation of yeast cells a slightly modified method according to Gietz was used (Gietz & Woods, 2006). Briefly, in the evening 20 ml of fresh media was inoculated with cells from a culture grown for 1-2 days. When the culture reached an OD_{600} of about 0.5 – 0.8 in the next day cells were harvested in a 50 ml falcon tube (3200 rpm, 3 min). Cell pellet was washed once in 10 ml of sterile ddH_2O, once in 1 ml of 100 mM LiAc (transferred in a 1.5 ml sterile eppendorf tube) and resuspended in 200 µl of 100 mM LiAc. For one transformation reaction 50 µl of the cell suspension was transferred to a 1.5 ml eppendorf tube, centrifuged at 13.000 rpm for 15 sec, the s/n was discarded and following reagents were added to the cell pellet (in this order):

240 µl	50% (w/v) PEG 3500 (autoclaved)
36 µl	1M LiAc (autoclaved)
10 µl	boiled ssDNA (10 mg / ml), 95°C for 5 min
12 µl	purified PCR product (from gel extraction)
or 1 µl	1:10 dilution of plasmid DNA from mini preparation
up to 360 µl	with ddH_2O

The mix was vortexed until pellet has solved and incubated in a water bath or thermo shaker at 42°C for 30-40 min. After a centrifugation step (3200 rpm, 3 min), the pellet was resuspended in 150 µl of sterile ddH_2O and the cell suspension was finally plated with sterile glass beads (∅ 3 mm) on selective media. Transformed yeast cells using KanMX as selection marker were not diluted in 3 ml of YPD (or in case of fas mutants in YPD+FA). Then, cells were incubated at 30°C for three to six hours on a shaker and finally plated on selective media.

4.3.8 Generation of yeast strains

Deletion and tagging of specific yeast genes were performed according to method described in (Guldener et al, 1996) and (Knop et al, 1999), respectively. Using respective oligonucleotides (see chapter 4.1.4) all gene deletions and epitope-tagged genes were verified by colony-PCR (see chapter 4.3.6) and / or Southern blot analysis (see chapter 4.3.10). In order to prove functionality of chromosomally epitope-tagged *FAS2*, corresponding strains expressing Fas1 and epitope-tagged Fas2 were tested for growth on solid- and in liquid media w/o fatty acid.

4.3.9 Southern blot analysis

In general the purpose of Southern blot analysis is to detect specific DNA sequences. In this study it was used to verify the correct *in vivo* recombination site of gene deletion cassettes into specific yeast genes. From a 5 ml o/n culture chromosomal DNA was isolated according to the protocol described in chapter 4.3.3. In a total volume of 25 µl, 10 µl of suspended DNA was digested with 20 Units restriction endonuclease in the corresponding restriction buffer o/n at 37°C. The next day 10 Units of the same restriction endonuclease was added and incubated for additionally two to three hours at 37°C. The digest was mixed with DNA sample buffer and separated onto a 0.8 % (w/v) agarose gel at 110 V for 1h. In order to determine the size of the separated DNA fragments 5 µl of 1Kb DNA ladder was also loaded into the gel. The agarose gel was then blotted onto a Hybond™ nylon membrane. First, a porous mat was soaked in double distilled water and placed into the blotting chamber. Second, a nylon membrane cut to size was pre-soaked for 1 min in 2xSSC buffer and placed on top of the mat. Third, the agarose gel was carefully placed on top of the nylon membrane. Finally the lid was closed tightly and a vacuum was generated (50 mbar) by a vacuum pump. The top of the gel was covered by denaturing buffer for 10 min, and then

treated with neutralization buffer for 10 min followed by 20xSSC buffer for 90 min. After disassembling the blotting chamber the nylon membrane was incubated for 5 min under UV-light to covalently link the DNA fragments to the membrane. In a clean glass tube the nylon membrane was pre-equilibrated with hybridization-buffer for 30 min at 55°C before adding the labeled DNA probes. After incubation o/n at 55°C the nylon membrane was washed twice with 100 ml of primary washing buffer for 10 min at 55°C and washed twice with 200 ml of secondary washing buffer for 5 min at RT. Finally, the membrane was incubated with detection reagent (about 5 ml) for 5 min, dried and exposed to an ECL-Hyperfilm for 1-2 h in the dark.

Denaturing buffer:	0.5 M	NaOH
	1.5 M	NaCl
Neutralization buffer:	0.5 M	Tris-HCl (pH 7.0)
	3 M	NaCl
20xSSC buffer:	0.3 M	$C_6H_7NaO_7$ (pH 7.0)
	3 M	NaCl
Hybridization buffer:	0.5 M	NaCl
	4 % (w/v)	Blocking reagent (Kit)

The hybridization buffer was stored in appropriate aliquots at – 20°C

Primary washing buffer:	2 M	Urea
(always freshly prepared)	50 mM	NaP pH 7.0
	150 mM	NaCl
	0.1% (w/v)	SDS
	1 mM	$MgCl_2$
	0.2 % (w/v)	Blocking reagent (Kit)
20x of the 2nd wash buffer:	1 M	Tris
	2 M	NaCl
	pH adjusted to 10.0	

Working solution: Dilute 1:20 and add $MgCl_2$ to give a final concentration of 2 mM

Labeled DNA probe: 100 ng purified DNA from gel extraction (5 min, 95°C)
+ 10 µl reaction buffer
+ 2 µl Labeling reagent
+ 10 µl Crosslinker working solution
Incubation for 30 min at 37°C
+ 1 µl Probe DNA for 1Kb DNA ladder (provided by R. Phillip)

4.3.10 Direct fluorescence microscopy

The fluorescence microscopy is an imaging technique making possible to observe fluorescing specimens. A confocal laser-scanning microscope was used analyzing the cellular distribution and localization of chromosomally C-terminal EGFP-tagged Fas2. Three OD_{600} of cells, grown to early log-phase, were collected by centrifugation for 3 min at 3100 rpm in a 50 ml falcon tube. The cells were washed once with 2 ml of PBS-IF buffer and resuspended in 2 ml of PBS-IF. For nuclei staining, 2 µl of Hoechst 33342 (10 mg / ml) was added to the cell suspension and incubated in the dark for 25 min at 30°C on a shaker (150 rpm). For cell fixation, 1 ml of the cell suspension was then transferred to a 1.5 ml eppendorf tube, 121 µl of 37% formaldehyde was added (drop by drop) to give a final concentration of 4% and incubated in the dark for 40 min at 30°C on a rotating wheel. Subsequently, the cells were washed twice with 1 ml of- and concentrated in 50 µl IF-buffer. 1-2 µl of fixed cells were spotted onto a microscope glass slide and mounted with a plastic cover slip as well as 3 µl of the mounting media mowiol. The sample was dried o/n in the dark at RT and finally sealed by nail polish. To visualize Hoechst 33342 and Fas2-EGFP by the confocal laser-scanning microscope (LSM710, Carl Zeiss) the fluorophores were excited according to their excitation wavelength with an argon laser (488 nm). Analysis of the obtained data and imaging were performed with the AxioVision Rel. 4.8.2 software (Carl Zeiss). Corresponding yeast strains with untagged Fas2 were used to determine background fluorescence.

PBS-IF buffer:	75 mM	NaCl
	53 mM	Na_2HPO_4
	13 mM	NaH_2HPO_4
IF-buffer	1.2 M	Sorbitol
	100 mM	KP_i (pH 6.5)

4.4 Biochemical and immunological methods

4.4.1 Alkaline lysis of yeast cells

2 OD_{600} of cells from an o/n culture were harvested by centrifugation at 13.000 rpm for 15 sec (table centrifuge was used for centrifugation steps). To remove residual media cell pellet was washed in 1 ml ddH_2O. Cells were resuspended in 1 ml ddH_2O and lysed with 150 µl of 1.85 M NaOH / 7.5% β-mercaptoethanol and kept for 15 min on ice. In between, cells were vortexed 2 to 3 times. In order to precipitate the proteins 150 µl of 50% TCA was added to the sample, mixed, and kept for 15 min on ice. The sample was centrifuged at 13000 rpm for 10 min at 4°C, the s/n was aspirated and the pellet was washed twice with 500 µl ice-cold acetone. After a further centrifugation step at 4°C the pellet was dried at 37°C for 10 min and then solubilized in 100 µl urea sample buffer at 37°C for one hour. Sample was boiled for 5 min at 95°C, centrifuged for 1 min at 13.000 rpm and 10 µl of the s/n was loaded onto a SDS gel.

Urea sample buffer:	8 M	Urea
	200 mM	Tris-HCl pH 6.8
	0.1 mM	EDTA
	5%	SDS
	0.03 %	Bromphenolblue
	1.5 % (v/v)	β-mercaptoethanol (always freshly added)

4.4.2 Native lysis of yeast cells by glass beads

150 OD_{600} cells grown to log-phase (about OD_{600} = 1.0) were harvested by centrifugation in 50 ml falcon tubes at 3200 rpm, 3 min, at 4°C. The pellets were washed in a total volume of 40 ml 30 mM ice-cold NaN_3 (thereby combined), resuspended in NaN_3 and stored for 10 min on ice. After a further centrifugation step, s/n was completely removed, the pellet immediately snap frozen in liquid nitrogen and stored at – 80°C. For starting the lysis, 150 OD_{600} of cells were thawed on ice and gently resuspended in 2 ml ice-cold extraction buffer (always freshly prepared including protease inhibitors and NP-40). All steps were carried out on ice or in the cold room (10°C). The cell suspension was split in 2x2 ml pre-chilled safe lock eppendorf tubes containing 1 ml acid washed glass beads. The lids of the tubes were sealed with parafilm and cells were vortexed 4 times for 4 min at 10°C with 1 min break on ice in

between. To separate cell debris from cell extract tubes were spun down at 500 x g for 5 min at 4°C. After transferring and combining the respective supernatants into a new 2 ml safe lock eppendorf tube centrifugation at 13.000 g for 20 min at 4°C was performed to obtain the cytosolic fraction (S13K) and the pellet fraction (P13K). S13K was transferred in a new 2 ml safe lock eppendorf and filled up to 2 ml with fresh prepared cold extraction buffer. For further analysis (e.g. pull down assay) protein concentration of cytosolic fraction was determined by Bradford assay and concentration differences between individual samples adjusted with extraction buffer. The P13K pellet was washed twice in 1 ml ice-cold extraction buffer (here combined), dried for 10 min at 37°C and solubilized in 2 ml urea sample buffer for one hour at 37°C in a thermo shaker with vigorous shaking (1400 rpm).

2x Extraction buffer:	20 % (v/v)	Glycerol
	0.1 M	Tris
	1 mM	EDTA pH 8.0
	0.4 M	NaCl
	Adjusted to pH 7.5 with HCl, buffer was autoclaved	

1x Extraction buffer (always freshly prepared):

½ Vol.	2x Extraction buffer
0.5% (v/v)	NP-40
1x	Protease Inhibitor Cocktail (in ddH_2O)
1 mM	PMSF (in isopropanol)
1.46 µM	PepstatinA (in methanol)
filled up to 1 Vol with ddH_2O	

1 Tablet of Protease Inhibitor Cocktail was dissolved in 2 ml of sterile ddH_2O resulting in a 25x stock solution

4.4.3 SDS-PAGE and Western blot analysis

SDS – PAGE

To separate protein samples according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, post-translational modifications and other factors) SDS–PAGE was performed. For this method, two different gels were generated, separating- and stacking gel. The gel composition is shown in Table 8. To avoid dehydration of the poured separating gel and to remove bubbles from top of the surface of the separating gel, it was covered with approximately 300 µl of isopropanol. As soon as the separating gel was

polymerized (at least after 45 min) the isopropanol was removed by rinsing with distilled water and the stacking gel was poured on top of the separating gel. Either a 10 well- or 15 well comb was placed into the stacking gel. After a polymerization time of at least 30 min gels were wrapped in wet tissue papers and stored in a plastic bag at 10°C for 1-2 days. The electrophoresis was carried out with 1 x SDS running buffer and a voltage of 100 – 150 volts for 1 to 2 hour(s). To determine the size of loaded proteins prestained protein marker was loaded into a separate pocket.

Components	9% separating gel	4% stacking gel
ddH_2O	8.7 ml	7.35 ml
1.5 M Tris/HCl pH 8.8	5 ml	--
1 M Tris/HCl ph 6.8	--	1.25 ml
10% (w/v) SDS	200 µl	200 µl
30% Acrylamid solution	6 ml	1.25 ml
10% APS	200 µl	200 µl
TEMED	10 µl	10 µl

Table 8: Composition of separating- and stacking gel for 2 gels

Blotting

Proteins are transferred from a SDS gel to a nitrocellulose membrane using a wet tank blot apparatus. The "sandwich" for the gel holder cassette was prepared in following order: one fibre pad, two whatman papers, gel, nitrocellulose membrane, two whatman paper, one fibre pad. "Sandwich" was assembled in an appropriate tray containing cold blotting buffer. Depending on the protein molecular weight the transfer was done as follow:

For proteins up to 150 KDa: t = 1.5 h I = 300 mA

For proteins above 150 KDa: t = 3.5 h I = 200 mA

During protein transfer tank was always kept on ice.

Transfer buffer:
25 mM Tris
192 mM Glycine
10% (v/v) Methanol
0.02% (w/v) SDS (only for proteins >150 KDa)
Storage at 10°C

Immunological detection

The nitrocellulose membrane was blocked for 1h at RT (or optional o/n at 10°C) with 5% skim milk (in 1xPBS-T) to prevent unspecific binding of the first and secondary antibody to the membrane. After washing with 20 ml PBS-T (3x10 min) the membrane was incubated with 20 ml of the primary antibody (1:10000 diluted in 1xPBS-T) directed against the antigen, which has to be detected, for 1h at RT or o/n at 10°C. To remove unbound primary antibodies the membrane was washed with 20 ml 1xPBS-T (3x10 min). The secondary antibody (1:10000 diluted in 1xPBS-T) directed against the first antibody was incubated for 1h at RT. The membrane was then washed 3 times for 10 min with 20 ml 1xPBS-T to remove unspecific bound secondary antibodies. As the secondary antibody is conjugated with horseradish peroxides (HRPO) it is possible to detect the corresponding protein sample using an ECL detection kit (ECL Western Blotting Substrate, Pierce). The membrane is incubated with a 1:1 mix of Lumi–Light Western Blotting Substrate 1 and 2 for about 1 min and an ECL film (Hyperfilm™ ECL, Amersham Biosciences) was exposed to the chemiluminescence emitted from the membrane. The exposition of the ECL film to chemiluminescence was performed for different times (for 30 sec, 1 min, 5 min or 15 min) to obtain an optimal signal.

Nitrocellulose membranes can be used for several times. After a first use and for each following one the membrane has to be stripped with 10% acetic acid in order to remove the antibodies from the membrane surface. To remove remaining acetic acid membrane has to be washed several times in distilled water.

1xPBST pH 7.4	100 mM	NaCl
	6 mM	Na_2HPO_4
	4 mM	NaH_2PO_4
	0.1% (v/v)	Tween 20

4.4.4 Determination of protein concentration by Bradford assay

In order to determine the protein concentration in solution Bradford assay was applied. Here, 100 µl of a 1:100 dilution (in ddH_2O) of a sample (e.g. cytosolic fraction after native cell breakage with glass beads) was added to 1 ml of Bradford reagent, mixed well, incubated for 5 min at RT and the optical density at 595 nm was determined. As reference served a 1:100 dilution (in ddH_2O) of the lysis buffer.

Bradford reagent:

100 mg of Coomassie Brilliant – Blue G-250 was dissolved o/n in 96% Ethanol.
100 ml of 85% phosphoric acid was added and filled up to 1L with distilled water
The solution was sterile filtrated and stored in the darkness at 10°C.

4.4.5 Cycloheximide chase analysis

This method is used to determine roughly a protein's half-life *in vivo*. Briefly, in the afternoon 20 ml of fresh media was inoculated with cells from a pre-culture grown to stationary phase. Next day, when the inoculated culture reached an OD_{600} of about 1.0 (log-phase) 8 OD_{600} of cells were harvested by centrifugation at 3200 rpm for 3 min. The s/n was discarded, pellet was resuspended in 2 ml fresh media and in order to stop protein synthesis cycloheximide was added to give a final concentration of 0.5 mg/ml (t=0h). Yeast cultures that carry a temperature sensitive allele were grown at 25-26° (permissive temperature) and, prior the addition of cycloheximide, shifted to 37°C (non-permissive or restrictive temperature) for 1h to inactivate the corresponding temperature sensitive protein. Cell suspension was incubated at 30°C (or at 37°C in case of temperature sensitive strains) on a shaker (150 rpm), 2 OD_{600} cells were collected at the indicated time points and added to 500 µl ice-cold 30 mM NaN_3. All collected samples were stored on ice until last sample was taken. Samples were centrifuged at 3200 rpm for 3 min at 4°C, s/n aspirated, pellets were stored at – 20°C or further processed. After one washing step with 1 ml ice-cold ddH_2O cell extracts were prepared by alkaline lysis (see chapter 4.4.1) and subjected to SDS-PAGE followed by immunodetection. After blocking in 5% skim milk (in PBST), nitrocellulose membrane was cut into two equal parts and each part was incubated with a different primary antibody (e.g. α-FAS and α-PGK).

4.4.6 Pulse chase analysis

In the mid of the 50's of the last century George Palade and his colleagues used pulse chase experiments to determine the roles of the rough endoplasmic reticulum and golgi apparatus in the production and secretion of proteins (Siekevitz & Palade, 1958). In a pulse chase experiment the progression of a radiolabeled molecule such as an amino acid is tracked through a cell. In addition to this, pulse-chase experiments

combined with immunoprecipitation allows also quantitative analysis of proteins in cells over time. Latter mentioned method was applied to determine the degradation characteristic of orphan Fas2 in different yeast mutants.

1st day: 10 ml of fresh YPD-FA (or YCM-FA + supplements) was inoculated with a single yeast colony fresh grown on solid media and incubated for 2 days at 30°C until they reached the stationary phase (pre-culture). Temperature sensitive mutants were grown at 25°C for 3 days.

3rd day: To setup the main-culture 20 ml of fresh media was inoculated with cells from the pre-culture.

4th day: 10 OD_{600} of logarithmic growing cells (OD_{600} between 0.8 and 1.2) were harvested by centrifugation at 3200 rpm for 3 min in a 50 ml falcon tube, washed 3 times with 1 ml and resuspended in 1 ml of fresh prepared and prewarmed 1xLabeling media. Cell suspension was then incubated in a shaking water bath (150 rpm) for 50 min at 30°C (starvation). The pulse phase begins when radioactive labeled methionine (^{35}S-methionine) is added to the cell's culture media. Thereafter, the radioactive amino acids are incorporated into the proteins translated during protein synthesis. In my case I used 20 µCi ^{35}S-methionine per 1 OD_{600} of cells taken from a ^{35}S-met stock (c_{stock} = 10 µCi / µl) and incubated the cells for further 20 min the water bath. The chase phase begins when a very large amount of non-radioactive methionine was added to the cell's culture media. From now on, no more proteins are translated that contain radioactive methionine. Here, 1 ml of prewarmed chase media (equally to labeling media, but contains additional methionine and BSA) was added to the samples, mixed well, and immediately 450 µl was taken out and added into 2 ml screw-capped eppendorf tubes, containing 50 µl ice-cold 110% TCA (w/v). TCA precipitates proteins in a cell homogenate and in cell's media culture. The sample was right away stored o/n at -80°C. In the same manner samples were taken out, when not otherwise indicated, at 2h, 4h and 6h after addition of chase media.

5th day: In order to prepare the radioactive labeled proteins for immunoprecipitation cells were lysed. In detail, the samples were thawed at 37°C for few minutes and spun down in at 13.000 rpm for 8 min (centrifugation steps during pulse chase experiments were always done in a table centrifuge). The radioactive s/n was removed by aspiration and the pellet was washed twice with ice-cold (-20°C) acetone to remove the TCA (13000 rpm, 8 min). During this procedure, it is important not to touch the pellet because it is smeary and can break. In order to evaporate remaining acetone residue the samples

were left under the hood for about 10 - 20 min. Then 100 µl of breaking buffer and 2/3 volume glass beads were added to the pellet that was then three times alternately incubated at 95°C and vortexed for 2 min. (Note: Prior the first heating / vortexing cycle; it is really important to release the cell pellet completely from the bottom of the tube. Otherwise the cell lysis will be incomplete). The resulting cell homogenate was spun down for few seconds (2000 rpm, 10 sec) to bring down material from the cap. Then 1 ml of IP buffer containing 40 µl of prepared protease inhibitor cocktail (1 tablet in 2 ml ddH_2O), 10 µl of 100 mM PMSF (dissolved in isopropanol) and 2 µl of PepstatinA (1 mg / ml methanol) was added to the sample and mixed thoroughly by vortexing. After centrifugation at 13000 rpm for 15 min, 900 µl of the s/n (no beads!) was added to 1.5 ml safe-lock eppendorf tube containing 10 µl of polyclonal α-FAS-Antibody / Glycerol (1:1 mix) and incubated, rotating, o/n at 4°C.

6th day: 80 µl of protein-A sepharose beads (5 mg sepharose / 80 µl IP buffer w/o Triton X100 / sample) was added to the sample and incubated for an additional 1-3 hours on a rotator at 4°C. The sample was spun down for 30 sec at 2000 rpm, the s/n was carefully removed by aspiration and the pellet was washed 2-3 times with 1 ml IP buffer. After the last washing step, the s/n was carefully removed with a long thin tip and the pellet was incubated with 60 µl of urea sample buffer. Sample was incubated on a thermal shaker at 95°C with 1000 rpm for 5 min. After a centrifugation step at 13.000 rpm for 1 min 10 µl to 20 µl of the s/n was loaded onto a 9% SDS gel and electrophoresis was carried out at 100 – 150 volt for 2 hours. The SDS gel was dried under vacuum at 65°C for 2h by a drying system (Fröbel, Laborgeräte). Quantification was accomplished using Storage Phosphor Screen (Amersham) and PhosphorImager™. When not otherwise indicated obtained data from at least three independent experiments were analysed using ImageQuant™ software (version 5.2). The program Excel was used to create mean values, standard deviations of the mean (SEM) and respective graphs. Pre- and main-cultures of temperature sensitive strains were grown at 25°C. The starvation step was carried out at 25°C and 10 min before pulse cells were shifted to 37°C.

2xLabeling media (pH 6.0)	3.4 g /l	Yeast nitrogen base w/o NH_4SO_4 and aa
	2.0 g/l	D–Glucose
	40 mg / l	Adenine, Uracil, Tryptophane, Histidine
	60 mg / l	Arginine, Tyrosine, Lysine, Leucine
	100 mg / l	Phenylalanine
	200 mg / l	Glutamic acid, Aspartic acid
	30 mg / l	Valine
	400 mg / l	Threonine
	800 mg / l	Serine

Labeling media was adjusted to pH to 6.0 using sodium hydroxide. Labeling media was sterile filtrated. Mostly used auxotrophic markers for plasmid selection as well as fatty acids were omitted and added before use. Final concentration of myristic acids and tween 40 is 0.03% (w/v) and 1%(v/v), respectively.

Chase media:	was prepared from ½ vol of 2xlabeling media, including	
	6 mg/ml	Methionine (sterile filtrated)
	2 mg/ml	BSA (sterile filtrated)
	filled up to 1 vol with sterile ddH_2O	
Breaking buffer:	50 mM	Tris-HCl (pH 7.5)
	6 M	Urea
	1%	SDS
	1 mM	EDTA
IP buffer:	50 mM	Tris-HCl (pH 7.5)
	150 mM	NaCl
	1.25% (v/v)	TritonX-100
	6 mM	EDTA

4.4.7 Trypsin sensitivity assay

This assay was used to determine the folding state of orphan Fas2 in comparison to Fas2. Not tightly folded- and unfolded proteins have the characteristic to be more susceptible for proteolysis in the presence of active proteases (Taniuchi & Anfinsen, 1969). 10 OD_{600} of cells from a culture grown to log-phase were harvested, washed two times in cold ddH_2O, snap frozen in liquid nitrogen and stored at – 80°C. Next day, after thawing on ice, cells were washed in 1 ml of chilled cytosolic buffer and disrupted in the presence of 600 µl cytosolic buffer + 400 µl glass beads for 5 min on a cell disruptor at 10°C. After a centrifugation step for 5 min at 500xg (4°C) to pellet cell debris 400 µl of the s/n was split into equal amounts in two eppendorf tubes. After adding 15 µl of trypsin (0.1 mg / ml 1 mM HCl) to give a final concentration of 7 µg / ml tube was incubated at 30°C in a thermal shaker. Samples were taken (45 µl) at the indicated time points, added to 1 ml of cold ddH_2O and precipitated immediately by adding 150 µl of

50% TCA (one ice, with occasionally vortexing). After last sample was taken and precipitated with TCA for at least 15 min, all samples were centrifuged at 13.000 rpm for 10 min at 4°C and pellet was washed twice with 500 µl of ice-cold acetone. After drying for 10 min at 37°C, pellet was solubilized in 50 µl of urea sample buffer for 30-60 min at the same temperature. For SDS–PAGE / Western blot analysis; sample was boiled for 5 min at 95°C, spun down at 13.000 rpm for 1 min and 10 µl of the s/n loaded onto a SDS gel followed by immunoblotting.

Cytosolic buffer:	20 mM	HEPES pH 7.4
	14% (v/v)	Glycerol
	100 mM	KOAc
	2 mM	MgOAc

Trypsin-solution: 0.1 mg / ml Trypsin (Acetylated) Type V-S (Sigma) dissolved in 1 mM HCl

4.4.8 TAP pull down assay

This assay is used to investigate protein-protein interactions. A protein of interest tagged with a TAP epitope, which consists of calmodulin binding peptide, a tobacco etch virus protease cleavage site and protein A, is purified sequentially by two independent affinity steps on IgG- and calmodulin-containing resins, respectively. In the first purification step the immobilized protein is specifically released by protease cleavage (TEV) and in the second step by lowering the calcium-dependent affinity of CBP to calmodulin. Here, Fas2-TAP was pulled down only by the first purification affinity step, using sepharose beads coated with IgGs. Fas2-TAP and the putative interaction partners were eluted either by boiling in urea sample buffer (denaturing conditions) or by cleavage of the epitope by TEV protease (native conditions). The eluted sample was TCA precipitated and subjected to SDS–PAGE followed by immunoblotting. 40 µl from the cell extract, obtained by native lysis with glass beads (see chapter 4.4.2), was taken as input, added to 1 ml of cold ddH_2O and precipitated immediately by adding 150 µl of 50% TCA. After 15 min incubation on ice with occasionally vortexing sample was centrifuged at 13.000 rpm for 10 min at 4°C and pellet was washed twice with 500 µl of ice-cold acetone. After drying for 10 min at 37°C, pellet was solubilized in 100 µl of urea sample buffer for 30-60 min at the same temperature. For SDS – PAGE / Western blot analysis; sample was boiled for 5 min at

95°C, spun down at 13.000 rpm for 1 min and 10 µl of the s/n loaded onto a SDS gel followed by immunoblotting.

For pull down of TAP-tagged Fas2, the remaining 1.80 ml of the cell extract (prepared like described in chapter 4.4.2) was transferred into a new 2.0 ml safe lock eppendorf tube, 100 µl of IgG Sepharose™ 6 Fast Flow slurry was added (previously prepared in a ratio of 1:1 in extraction buffer) and incubated on a rotating wheel at 10°C for 2 – 3 hours. The beads were thoroughly washed three times with 1 ml fresh prepared, chilled extraction buffer (2000 rpm, 1 min, 4°C). After the last washing step remaining liquid residue was carefully removed with a long white tip, beads were boiled in 60 µl urea sample buffer for 5 min at 1050 rpm in a thermo shaker (denaturing conditions), spun down for 1 min at 13.000 and 10 µl analyzed by SDS – PAGE followed by immunoblotting.

Elution of Fas2-TAP under native conditions was performed to avoid the appearance of an unspecific band that runs almost at the same height with epitope-tagged Ubr1 (Ubr1-HA and Flag-Ubr1) and Fas2 (more details in see results). Here, after the three washing steps with chilled extraction buffer beads were washed two times with 1 ml of fresh prepared, chilled TEV Cleavage buffer (TEV-CB) each and incubated with 3 µl TEV – Protease (8 U / µl) in 100 µl TEV-CB for 2 – 3h at 16°C in a thermo shaker (1100 rpm). Beads were spun down (2000 rpm, 1 min, 4°C) and the s/n was collected in a new eppendorf tube stored on ice. To wash out remained Fas2-TAP plus putative interaction partners, beads were washed once with 400 µl of TEV-CB for 10 min at 30°C in a thermo shaker (1100 rpm), retrieved s/ns were combined and precipitated with 60 µl of 110% (w/v) TCA for 20 min on ice. After two washing steps with ice-cold acetone pellet was dried for 10 min at 37°C and solubilized in 50 µl urea sample buffer for 30-60 min at 37°C in a thermo shaker (1100 rpm). The sample was then boiled for 5 min at 95°C, spun down for 1 min at 13.000 and 10 µl analyzed by SDS–PAGE followed by immunoblotting.

4.4.9 Glycerol step density gradient centrifugation

Glycerol step density gradient centrifugation was performed according to (Kim et al, 1997). 50xOD_{600} of cells, grown to exponential growth phase, were harvested, centrifuged at 3100 rpm for 3 min and washed once in 20 ml of ice-cold sterile ddH_2O. When not further processed, cell pellet was stored right away at -80°C. After thawing on

ice, pellet was resuspended in 520 µl ice-cold 0.1 M KP_i buffer (pH 6.9), ice-cold 280 µl 25x Protease Inhibitor Cocktail (1 tablet solved in 2 ml sterile ddH_2O), 100 µl 0.1 M PMSF (solved in isopropanol) and 10 µl of 1.45 mM PepstatinA (solved in methanol). Cell suspension was added to a pre-chilled 2.0 ml safe-lock eppendorf tube containing 300 µl. The lid of the tube was sealed with parafilm and cells were vortexed 2 times for 10 min at 10°C with 1 min break on ice in between. To separate cell debris from cell extract tube was spun down at 500 x g for 5 min at 4°C. After transferring supernatant into a new 1.5 ml eppendorf tube a further centrifugation step was carried out at 13.000 rpm for 20 min at 4°C. Protein concentration of the retrieved supernatant (S13K) was determined by Bradford assay. Subsequently, protein concentration of different strains were adjusted. Then, 200 µl of S13K was layered on top of a glycerol step gradient (400 µl of each 50%, 40%, 30%, 20%, 10% glycerol in 20 mM PIPES buffer pH 6.8) prepared at 10°C in an open-top polyallomer tubes (Beckman Coulter). Sample was centrifuged for 4 hours at 55.000 rpm (269.000 x g) at 15°C in a TLS55 rotor (Beckman Instruments) without brake usage during the deceleration of the rotor. Subsequently, 170 µl fractions were collected from the top of the glycerol step gradient and precipitated with 17 µl of 110% (w/v) TCA for 10 min on ice followed by at least 30 min at -80°C. After thawing at RT, samples were spun down at 13.000 rpm for 10 min at 4°C and washed once with 1 ml of ice-cold acetone. Pellets were air-dried for 10 min at 37°C and solved in urea loading buffer for 30 min at 37°C and 1000 rpm in a thermoshaker. Protein samples were stored at -20°C. After thawing at RT, samples were boiled for 3-5 min at 95°C and 1000 rpm in a thermoshaker. 10 µl of each sample was subjected to SDS-PAGE followed by immunoblotting using specific antibodies

4.5 Databases und Software

Following protein-protein interaction databases were used looking for new components that are eventually involved in the degradation process of orphan Fas2:

http://thebiogrid.org/

http://chaperonedb.ccbr.utoronto.ca/index.jsp

Software used in this study is given in Table 9.

Used for	Program(s)
Processing of DNA sequences	A plasmid editor v2.0.44
Quantification of degradation kinetics obtained from pulse chase analysis	ImageQuant v5.2
Quantification of degradation kinetics obtained from cycloheximide chase analysis	ImageJ v1.46
Image processing and editing from Confocal Laser Scanning Microscopy	Carl Zeiss AxioVision 4.8.2
Image processing	Adobe Photoshop CS5 12.1 Adobe Illustrator CS5 15.1.0
Preparation of text, tables and diagrams	Microsoft Office 2008
Management of references	Endnote X4

Table 9: Software

5 Results

All components of the cell that contribute to folding of proteins into their native conformation, prevent protein aggregation or are involved in the degradation of terminally misfolded proteins are part of the protein quality control (PQC). These include mainly molecular chaperones, molecular motors and the ubiquitin–proteasome–system. Their involvement in PQC has been described for different cell compartments (Buchberger et al, 2010; Gardner et al, 2005; Langer & Neupert, 1996; Stolz & Wolf, 2010; Vembar & Brodsky, 2008; Voos, 2009). By using several different cytosolic model substrates in yeast, including mislocalized proteins (Heck et al, 2010; Park et al, 2007) thermosensitive mutant proteins (Kaganovich et al, 2008; Khosrow-Khavar et al, 2012) truncated protein versions (Heck et al, 2010; Prasad et al, 2010) substrates fused to a degron (Metzger et al, 2008) as well as a heterologously expressed orphan protein (McClellan et al, 2005) revealed different needs of chaperones, E2 enzyme(s) and E3 ligase(s) for their quality control and proteasomal degradation. So far, no physiological substrate without any mutation or truncation has been used to study CytoQC in yeast. I used a naturally occurring protein, the Fas2 subunit of the yeast fatty acid synthase to study CytoQC. Six Fas2- and six Fas1 subunits forming a very large 2.6 MDa fatty acid synthase macromolecule, which is responsible for synthesizing 16- and 18-carbon fatty acids (Schweizer and Hofmann, 2004; Lomakin, Cell 2007). Several years ago it has been reported that in the absence of one of the Fas subunits the corresponding partner subunit, which then presents an innate orphan protein, is suddenly susceptible to proteolytical degradation (Schüller, 1992; Egner, 1993). Whereas orphan Fas1 is targeted to vacuolar-mediated degradation, the 26S proteasome is required for the elimination of orphan Fas2. To understand in more detail how the cellular quality control and elimination system recognizes an innate orphan protein and guides it to the proteasome for degradation I followed the proteolytical fate of orphan Fas2 in *FAS1* deletion mutant (see Figure 3) and elucidated the components and the mechanisms involved in its quality control and degradation process.

5.1 Characterization of the model substrate orphan Fas2

5.1.1 Orphan Fas2 is proteolytically susceptible *in vitro* and is a substrate of the 26S proteasome *in vivo*

An *in vitro* trypsinization assay was used determining whether orphan Fas2 is a tightly folded- or a loosely folded protein. Whereas tightly folded proteins are strongly resistant to proteolytic enzymes unfolded or loosely folded proteins are more susceptible to proteolytic degradation (Taniuchi & Anfinsen, 1969). In an *in vitro* trypsinization assay, a cell lysate obtained by cell breakage with glass beads and without the use of protease inhibitors was split into two aliquots. One aliquot was treated with trypsin while the other was not. As shown in Figure 4 (right panel), the Fas subunits, Fas1 and Fas2, in a cell lysate obtained from wild type cells are not degraded over period of 30 minutes in the presence of trypsin whereas orphan Fas2 is very sensitive regarding its access to the proteolytic activity of trypsin and disappears in less than 10 minutes. In untreated cell lysates (Figure 4, left panel) the band of orphan Fas2 at time point zero is much sharper, but disappears, in contrast to the wild type, almost completely in less than 20 minutes. During glass beads mediated cell breakage without any protease inhibitors, intracellular proteases, especially the vacuolar proteinases yscA and yscB (Teichert et al, 1989), remain active and digest all proteins that are susceptible to their proteolytic activity (e.g. orphan Fas2). 3-phosphoglycerate-kinase (PGK) and fully assembled fatty acid synthase are, at least over a time period of 30 minutes, stable (Figure 4). Despite different molecular masses (Fas1: 229 KDa; Fas2: 207 KDa) both Fas subunits run almost at the same height (about 250 KDa) in a SDS gel and are recognized on an immunoblot by the same anti FAS antibody (Egner et al, 1993). Thus, the large band in the lanes for wild type (WT) consists of two bands, the upper band is Fas2 and the lower band is Fas1; see also (Egner et al, 1993; Schuller et al, 1992). PGK served as positive control for a tightly folded protein.

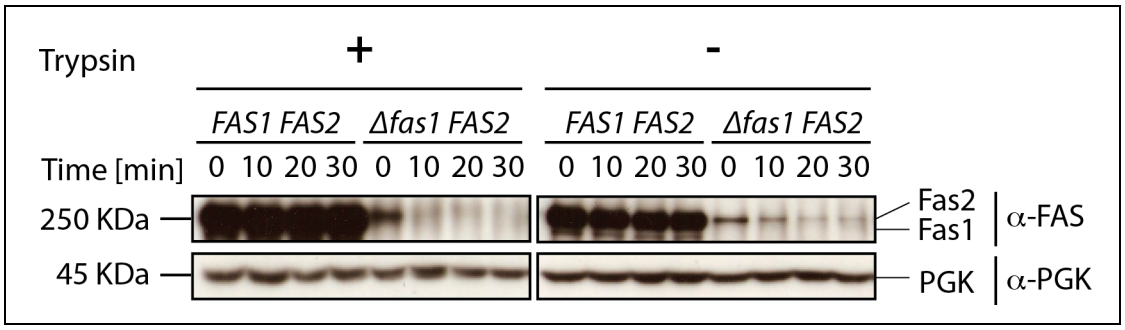

Figure 4: **Orphan Fas2 is sensitive to proteolytic enzymes.** Native cell breakage of wild type (YWO 0903)- and Δ*fas1* (YMS 5) cells was performed with glass beads and without protease inhibitors. Cell debris is spun down by a 500 x g centrifugation step and the lysate was split into two aliquots. To one aliquot trypsin was added (final concentration: 7 µg / ml), the other aliquot without trypsin served as negative control. The lysates were incubated at 30°C, samples were taken at the indicated time points and precipitated with TCA. Samples were subjected to SDS PAGE followed by immunoblotting. The immunoblot was cut into two parts and incubated with FAS- and PGK antibody, respectively. FAS-antibody recognizes Fas1 and Fas2 (Egner et al, 1993). Note that expression of Fas2 is decreased in the *FAS1* deletion strain (Wenz et al, 2001).

In yeast wild type cells, six Fas1- and six Fas2-subunits are assembled to a multienzyme complex forming the barrel-shaped fatty acid synthase (FAS), which is responsible for the synthesis of long chain fatty acids (Lomakin et al, 2007). Whereas the assembled FAS-complex is proteolytically stable *in vivo* the unassembled Fas2-subunit (orphan Fas2) becomes a substrate of the 26S proteasome (Egner et al, 1993). This finding was confirmed using the conditional yeast mutant *cim3-1*, defective in the Rpt6 subunit of the proteasome cap, and deleted in *FAS1*. Degradation of orphan Fas2 was followed by cycloheximide chase analysis at the restricted temperature of 37°C (see chapter 4.4.5). In contrast to the proteasome competent *FAS1* deletion strain (*CIM3 Δfas1*), in which orphan Fas2 is degraded with a *in vivo* half-life of about 2 hours, the model substrate is almost completely stabilized in the proteasomal mutant *cim3-1 Δfas1* (Figure 5). To check whether orphan Fas2 is subjected to vacuolar mediated degradation I tested its fate in a *FAS1* deletion strain in the presence or absence of two important vacuolar proteases, proteinase yscA (Pep4) and proteinase yscB (Prb1). As shown in Figure 5, the absence of proteinase yscA and yscB *in vivo* has no significant influence on the degradation process of orphan Fas2, confirming the result that this subunit is mainly targeted to the 26S proteasome for degradation.

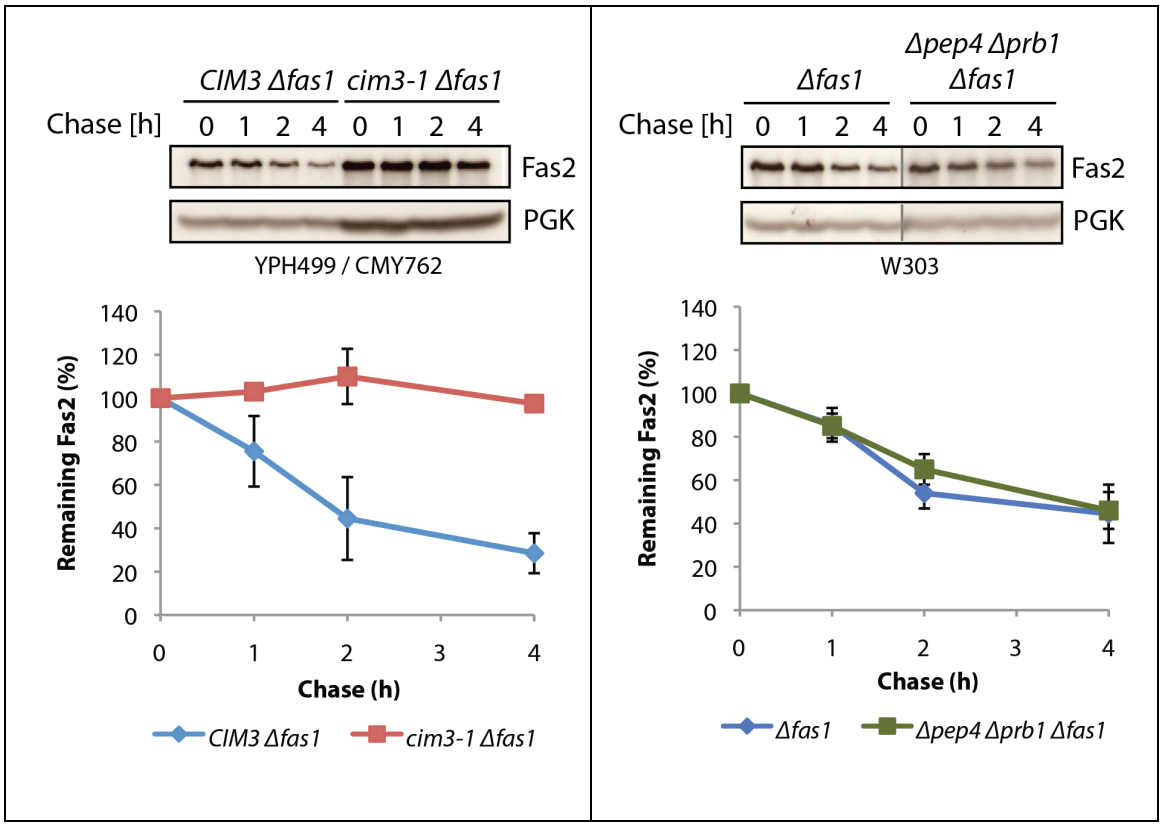

Figure 5: **Orphan Fas2 is degraded by the 26S proteasome.** Cycloheximide chase analyses of orphan Fas2 in the thermosensitive proteasome mutant *cim3-1 Δfas1* (YMS 7) (left panel), in the *Δpep4 Δprb1 Δfas1* (YMS 68) deletion strain (right panel) and in the corresponding wild type strains, CIM3 *Δfas1* (YMS 186) and *Δfas1* (YMS 5) were performed. Before adding cycloheximide (t=0h) to the *cim3-1 Δfas1* mutant (YMS 7) and its corresponding control strain *CIM3 Δfas1* (YMS 186), cells were incubated at non-permissive temperature (37°C) for one hour. Samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control. Band intensities of Fas2 were quantified with ImageJ and plotted as graph using Excel. Plotted data represent the mean values of two independent experiments. Error bars represent the standard deviation of the mean. Additional lanes in the immunoblot (right panel) were removed, in which another deletion mutant was shown. This is indicated with a grey vertical line. YPH499 / CMY762 and W303 are official names for different strain backgrounds, in which YPH499 is the congenic wild type strain of CMY762 (Ghislain et al, 1993).

5.1.2 Orphan Fas2-EGFP is localized to the cytosol

Due to recent studies showing that misfolded cytosolic proteins can be targeted to the nucleus for proteasomal degradation (Heck et al, 2010; Prasad et al, 2010), it was important to know whether orphan Fas2 is localized exclusively to the cytosol or it is also imported into the nucleus. In S288C yeast wild type cells, C-terminally GFP tagged Fas2 is localized to the cytosol (Huh et al, 2003). To assess the localization of Fas2 as well as orphan Fas2 in the W303 strain background, the chromosomal *FAS2* ORF was C-terminally tagged with yeast-enhanced green fluorescent protein (EGFP) according to the method of Knop et al., 1999. EGFP is a GFP variant optimized for expression in fungi (Cormack et al, 1997). In order to prove whether the chromosomally expressed Fas2-EGFP fusion protein is still functional regarding to fatty acid synthesis the corresponding yeast mutant (W303-1B *FAS2-EGFP*) was tested for the ability to grow on rich solid- and in rich liquid media without fatty acid supply (YPD). Using the wild type strain of W303-1B as positive control, it was observed that the W303-1B *FAS2-EGFP* mutant was able to grow on rich solid media and in addition has the same doubling time (90 min) in rich liquid media as wild type, making obviously that the EGFP epitope has no critical influence on Fas2 with respect to fatty acid synthesis and cell division time (data not shown). Fas2-EGFP- and orphan Fas2-EGFP localization was determined by fluorescence microscopy using a Zeiss laser-scanning microscope. Consistent with the previous found Fas2 localization results in the S228C strain background (Huh et al, 2003), Fas2-EGFP fluorescence (green) in wild type cells of W303 shows a scattered pattern throughout the cytosol (Figure 6). A similar EGFP fluorescence pattern was found for cells expressing orphan Fas2-EGFP. No EGFP fluorescence is visible in the nuclei of the *FAS2-EGFP*- and the *Δfas1 FAS2-EGFP* mutant strains, which were co-stained with Hoechst 33342 (red fluorescence). This points to the assumption that orphan Fas2 is still localized to the cytosol. The weaker fluorescence signal of orphan Fas2-EGFP (Δ*fas1* cells) in contrast to Fas2-EGFP (wild type cells) is explained by the fact that the Fas2 expression level is decreased in a *FAS1* deletion strain (Wenz et al, 2001). Yeast strains (wild type and *Δfas1*) expressing untagged Fas2 (no EGFP) were used as negative control for the EGFP fluorescence (Figure 6).

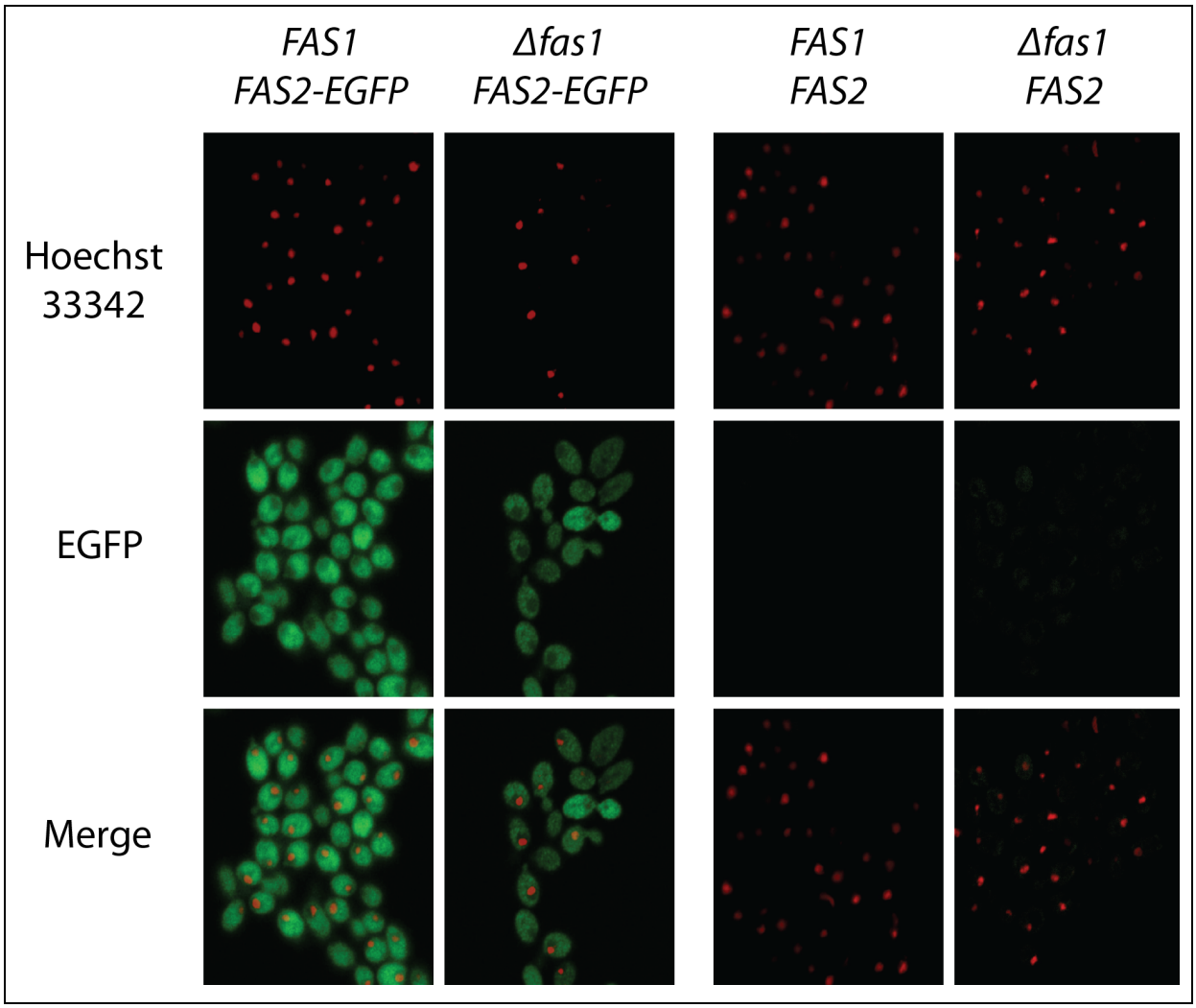

Figure 6. **Orphan Fas2 is localized to the cytosol.** Wild type cells expressing endogenous Fas2 (YWO 0903) or Fas2-EGFP (YMS 180) and Δ*fas1* cells, expressing endogenous Fas2 (YMS 5) or Fas2-EGFP (YMS 131) were grown to early exponential growth phase in YPD at 30°C (strains lacking *FAS1* were grown in YPD-FA). Equal amounts of cells were collected by centrifugation, washed and resuspended in PBS-IF buffer. Cell nuclei were stained by incubation with Hoechst 33342 followed by cell fixation with formaldehyde. Cells were mounted on a microscope slide and analyzed under a laser-scanning microscope (LSM710, Carl Zeiss) using Hoechst 33342- and EGFP filter. Images and overlays were done using AxioVision Rel. Software.

5.1.3 In comparison to orphan Fas2, the *in vivo* half-life of C-terminally EGFP tagged orphan Fas2 is decreased

It is commonly known that an epitope fused to a protein can alter its *in vivo* half-life, and/or affect its functionality (Yewdell et al, 2011). Last mentioned concern regarding cellular growth can be excluded for Fas2-EGFP (see chapter 5.1.2). To compare the degradation rates of orphan Fas2 and orphan Fas2-EGFP, cycloheximide chase experiments were performed using respective yeast mutants. It turned out that the C-terminally fused EGFP epitope dramatically increased the turnover rate of orphan Fas2-EGFP (Figure 7). Whereas the degradation of untagged orphan Fas2 is characterized with an *in vivo* half-life of about 2 hours the Fas2-EGFP orphan shows a half-life of about 30 min and disappears almost completely after 60 min.

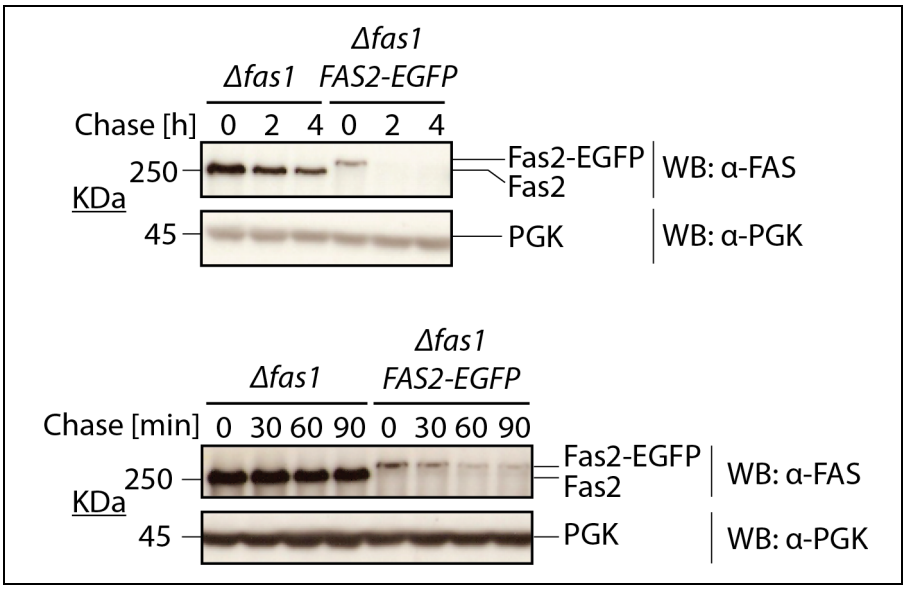

Figure 7. **Orphan Fas2-EGFP is much faster degraded than orphan Fas2.** Cycloheximide chase analyses of endogenously expressed orphan Fas2 (YMS 5) and orphan Fas2-EGFP (YMS 131) in a *FAS1* deletion strain were performed. Cycloheximide was added (t=0h) to exponentially grown cells, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control.

5.1.4 Studies on orphan Fas2 stoichiometry

In yeast wild type cells, the 2.6 MDa fatty acid synthase consists of six copies each of Fas2 (M_r = 207 KDa) and Fas1 (M_r = 229 KDa) subunits forming a large barrel-shaped macromolecule. Here, the six Fas2 subunits are organized in a wheel-like structure covered by three Fas1 subunits each on top and bottom of the wheel (Lomakin et al, 2007). As I worked with a *FAS1* deletion mutant the question was open whether the remaining Fas2 orphan proteins are still assembled into a wheel-like structure (Fas2 hexamer), which should have a molecular weight of approximately 1.2 MDa, or whether they do exist only as Fas2 monomers. In order to investigate this issue glycerol step gradient density centrifugation experiments (see chapter 4.4.9) with native cell extracts prepared from a *Δfas1*- and, as a control, from the corresponding wild type strain were done. As marker proteins served glucose-6-phosphate dehydrogenase (G6PDH, tetramer of 240 KDa (Saliola et al, 2007)), an AAA ATPase protein (Cdc48, hexamer of 540 KDa) and fatty acid synthase (Six Fas2 plus six Fas1 subunits forming a dodecamer of 2.6 MDa). As seen in Figure 8B orphan Fas2 sediments in fraction 4 to 12, with a maximum in fractions 9 to 11. The 2.6 MDa fatty acid synthase (Figure 8A) sediments mainly in fraction 12, the 540 KDa Cdc48 tetramer mainly in fraction 8 and 9, the 240 KDa G6PDH tetramer mainly in fraction 3 to 6 (Figure 8A and B). According to the sedimentation pattern of the three marker proteins in a 10-50% glycerol gradient most of the orphan Fas2 proteins (fraction 9 to 11) sediment at a high molecular mass fraction, ranging between more than 540 KDa and less than 2.6 MDa, suggesting that most of those orphan proteins are most likely organized in an assembly structure of a 1.2 MDa homohexamer. The weakly detectable amount of orphan Fas2 in fraction 4 to 5, which co-sediments with the 240 KDa G6PDH marker and the Cdc48 marker may be due to the existence of small amounts of orphan Fas2 monomers and assembly intermediates. However, it cannot be completely excluded that the sedimentation pattern of orphan Fas2, ranging from fraction 4 (~ 240 KDa) to fraction 8 (~ 540 KDa), is due to native orphan Fas2 interaction partners involved in FAS biogenesis or in degradation of the orphan Fas2 protein.

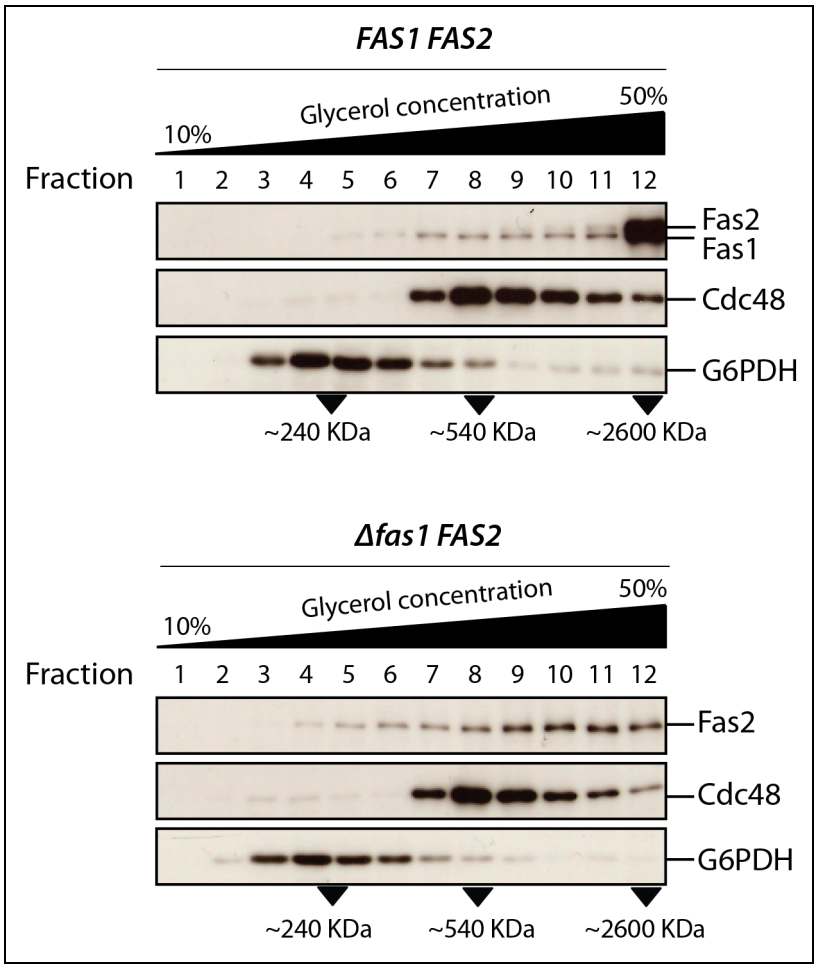

Figure 8. **Glycerol step density gradient centrifugation.** Native cell extracts prepared from wild type- (YWO 0903) and Δ*fas1* (YMS 5) cells and were centrifuged in a 10-50% glycerol step density gradient to separate proteins according to their sedimentation coefficient. 12 fractions were collected, beginning from top of the gradient (fraction 1), and proteins were TCA precipitated and subjected to SDS-PAGE followed by immunoblotting. Fatty acid synthase (Dodecamer M_R=2.6 MDa), consisting of six Fas1- and six Fas2-subunits, and orphan Fas2 (207 KDa) were detected with FAS antibody. Cdc48 (Homohexamer: M_r=540 KDa) and G6PDH (Homotetramer: M_r=240 KDa) were detected with Cdc48- and G6PDH antibody, respectively.

5.2 The role of chaperones and co-chaperones in protein quality control of orphan Fas2

Molecular chaperones are in involved in different processes within living cells, including protein folding, translocation of proteins across membranes, signal transduction and even protein degradation (Arndt et al, 2007; Hartl & Hayer-Hartl, 2002). In terms of protein quality control, which takes places in different cell compartments, (e.g. the endoplasmic reticulum, the nucleus, mitochondria and in the cytosol), molecular chaperones fulfil the need to protect newly synthesized proteins against (self)-aggregation, to assist correct folding of non-native proteins and to refold already aggregated or misfolded proteins back to their native conformation. The first hints of a direct participation of chaperones in protein degradation came from studies investigating the mechanisms of endoplasmic reticulum associated protein degradation (ERAD) (Arndt et al, 2007; Stolz & Wolf, 2010; Vembar & Brodsky, 2008). In the last 10 years it became increasingly clear that chaperones play also an active role in promoting degradation of terminally misfolded proteins in the cytosol. Recent studies have revealed that the degradation process of diverse cytosolic model substrates is delayed or even blocked in different cytosolic chaperone defective mutants. Mutated protein members of the Hsp70-, Hsp40-, Hsp90 or Hsp110 chaperone family disturb the degradation of cytosolic misfolded proteins (Eisele, 2011; McClellan et al, 2005; Metzger et al, 2008; Park et al, 2007; Prasad et al, 2010). Consequently, it was important to clear the question whether the degradation of an orphan protein, orphan Fas2, requires the assistance of diverse molecular chaperones, too.

5.2.1 The Hsp70 chaperone Ssa1 is essential for the degradation of orphan Fas2

The four Ssa proteins, Ssa1 to Ssa4, belong the to Hsp70 chaperone family and share more than 80% sequence identity, Ssa1 and Ssa2 show even 98% identity. Despite a few exceptions (Brown et al, 2000; Schwimmer & Masison, 2002) Ssa proteins are functionally redundant. To prove whether Ssa chaperones are involved in cellular protein degradation in yeast, it is common to use a *SSA2*, *SSA3*, *SSA4* triple deletion strain that harbours in addition a thermosensitive allele of *SSA1* (*ssa1-45*). The thermosensitive *ssa1-45* allele encodes a P417L mutation in the peptide-binding domain of Ssa1 compromising protein folding, translocation and degradation at restrictive temperature (37°C) (Becker et al, 1996; Kim et al, 1997; Park et al, 2007; Zhang et al, 2001). As a control a *SSA2*, *SSA3*, *SSA4* triple deletion strain with an *SSA1* wild type allele was used. As described for temperature sensitive mutants in chapter 4.4.5, cycloheximide chase experiments were performed with wild type cells and both Ssa mutant strains (*SSA1 Δssa2 Δssa3 Δssa4* and *ssa1-45 Δssa2 Δssa3 Δssa4*), all in addition lacking *FAS1*, to follow the degradation of orphan Fas2. As seen in Figure 9, degradation of orphan Fas2 progresses during the chase of 4 hours in the *Δfas1*- and the *SSA1 Δssa2 Δssa3 Δssa4 Δfas1* mutant strain at both temperatures (25°C and 37°C). The same degradation rate of orphan Fas2 is also observed in the *ssa1-45 Δssa2 Δssa3 Δssa4 Δfas1* mutant strain at 25°C. However, under restrictive conditions (37°C) the elimination of orphan Fas2 in the *ssa1-45 Δssa2 Δssa3 Δssa4 Δfas1* mutant is almost completely abolished. Thus, it can be assumed that the Hsp70 chaperone Ssa1 is involved in the elimination process of the orphan substrate Fas2. To confirm the data obtained by cycloheximide chase analysis, pulse chase experiments with the same mutant strains were done. After several attempts I was not able to detect any radioactive signal of Fas2 on the Storage Phosphor Screen (Amersham) using PhosphorImager™ for the two strains *SSA1 Δssa2 Δssa3 Δssa4 Δfas1* and *ssa1-45 Δssa2 Δssa3 Δssa4 Δfas1*.

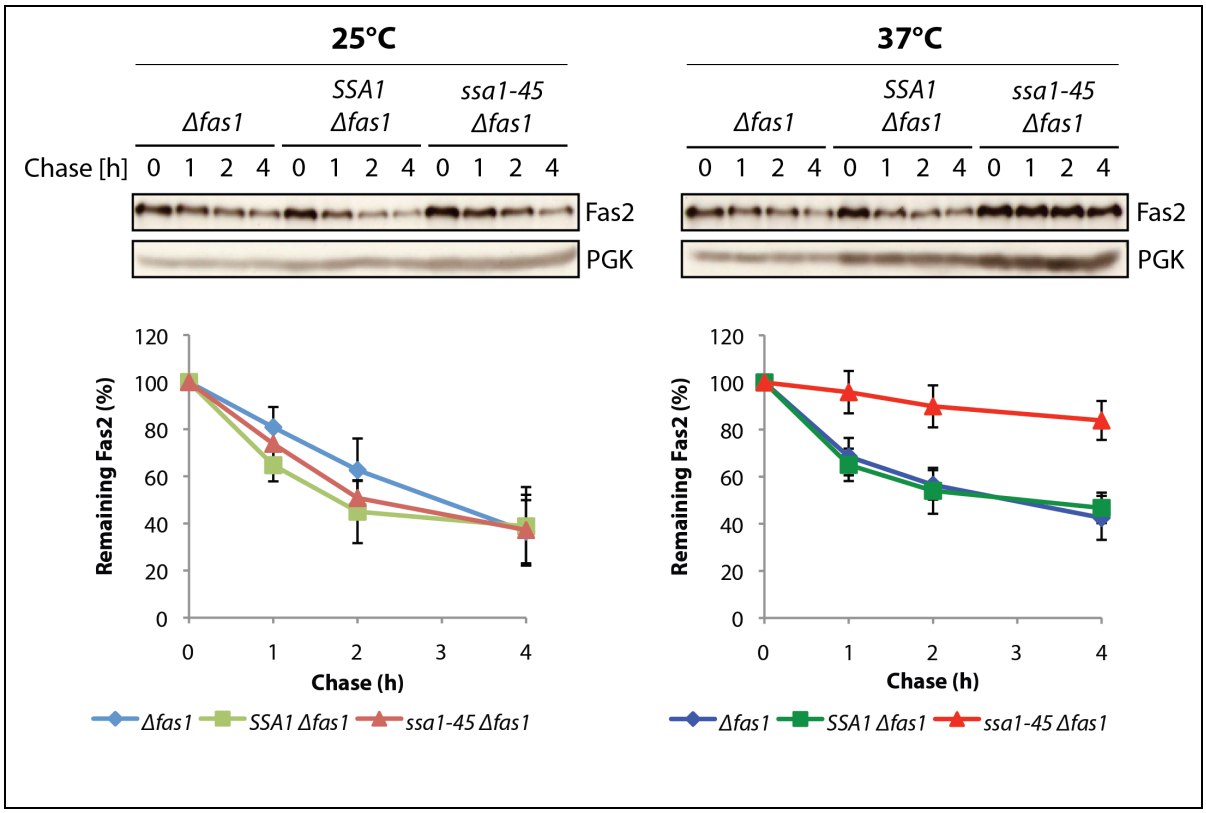

Figure 9. **The Hsp70 chaperone Ssa1 is required for orphan Fas2 degradation.** Cycloheximide chase analyses of orphan Fas2 in *Δfas1* (YMS 5) – , *SSA1 Δssa2 Δssa3 Δssa4 Δfas1* (YMS 35) – and *ssa1-45 Δssa2 Δssa3 Δssa4 Δfas1* (YMS 36) cells were performed according to the protocol for experimentation of temperature sensitive yeast strains (see chapter 4.4.5). Cycloheximide was added one hour after incubation at 25°C or 37°C. Samples were collected at the indicated time points. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control. Band intensities of Fas2 were quantified with ImageJ and plotted as graph using Excel. Plotted data represent the mean values of four independent experiments. Error bars represent the standard deviation of the mean.

5.2.2 Influence of Hsp40 chaperone(s) and nucleotide exchange factor(s) on Ssa1 mediated degradation of orphan Fas2

The Hsp70 reaction cycle comprises ATP- and client substrate binding to Hsp70, hydrolysis of ATP resulting in trapping of the substrate in the peptide binding cleft, replacement of ADP with ATP and substrate release (see chapter 3.1.1). Whereas the Hsp40 chaperones are able to specifically deliver the client protein to the respective Hsp70 and to stimulate the ATP hydrolysis in the Hsp70 reaction cycle, the nucleotide

exchange factors are responsible for rapid ADP/ATP exchange and release of the client substrate.

A typical Hsp70 co-chaperone is the Hsp40 member Ydj1 that is involved in the Ssa1 mediated degradation of some short-lived and abnormal proteins (Lee et al, 1996) as well as in degradation of cytosolic misfolded model substrates (Metzger et al, 2008; Park et al, 2007; Prasad et al, 2010). I therefore assessed whether Ydj1 plays a role in orphan Fas2 degradation. In addition two further members of the Hsp40 chaperone family. Xdj1, which is closely related to Ydj1, and Apj1, which was found to interact with Fas2 in yeast wild type cells (Krogan et al, 2006) were tested. The temperature sensitive yeast mutant *ydj1-151*, deleted in *FAS1*, was used following the degradation of orphan Fas2 under permissive- (25°C) and non-permissive conditions (37°C) in a pulse chase experiment. As is shown in Figure 10, orphan Fas2 degradation is not affected in the *ydj1-151 Δfas1* mutant under non-permissive conditions compared to permissive conditions, indicating that Ydj1 is not involved in the Ssa1 dependent degradation of orphan Fas2. The *FAS1* deletion strain served as control and exhibits a similar degradation manner at both conditions (25°C and 37°C). Similar results were obtained with *Δxdj1 Δfas1* (Figure 11) and *Δapj1 Δfas1* deletion strains at 30°C (Liane Schuster, Studienarbeit, 2010). Consequently, the Hsp40 chaperones Xdj1 and Apj1 seem to play no role in the degradation process of orphan Fas2. Recent studies have reported an active involvement of the Hsp110 chaperones Sse1 and / or Sse2, which are able to act as nucleotide exchange factors for Ssa chaperones, in cytosolic protein quality control (Eisele, 2011; McClellan et al, 2005; Prasad et al, 2010). Here, the degradation of cytosolic misfolded proteins was delayed or even blocked in mutants lacking Sse chaperone activity. As the double deletion of *SSE1 SSE2* is lethal (Raviol et al, 2006), I used a temperature sensitive *sse1* mutant, deleted in *SSE2* as well as in *FAS1*, and monitored the degradation of orphan Fas2 at permissive- (30°C) and non-permissive temperature (37°C). The corresponding *Δfas1* deletion strain served as control. As shown in Figure 12, the degradation rate of orphan Fas2 in the Δ*fas1* and in the *sse1-ts* Δ*sse2* Δ*fas1* mutant strain is not significant alternated at both temperatures, 30°C and 37°C. This suggests that Sse1 and Sse2 are not required for the elimination of orphan Fas2.

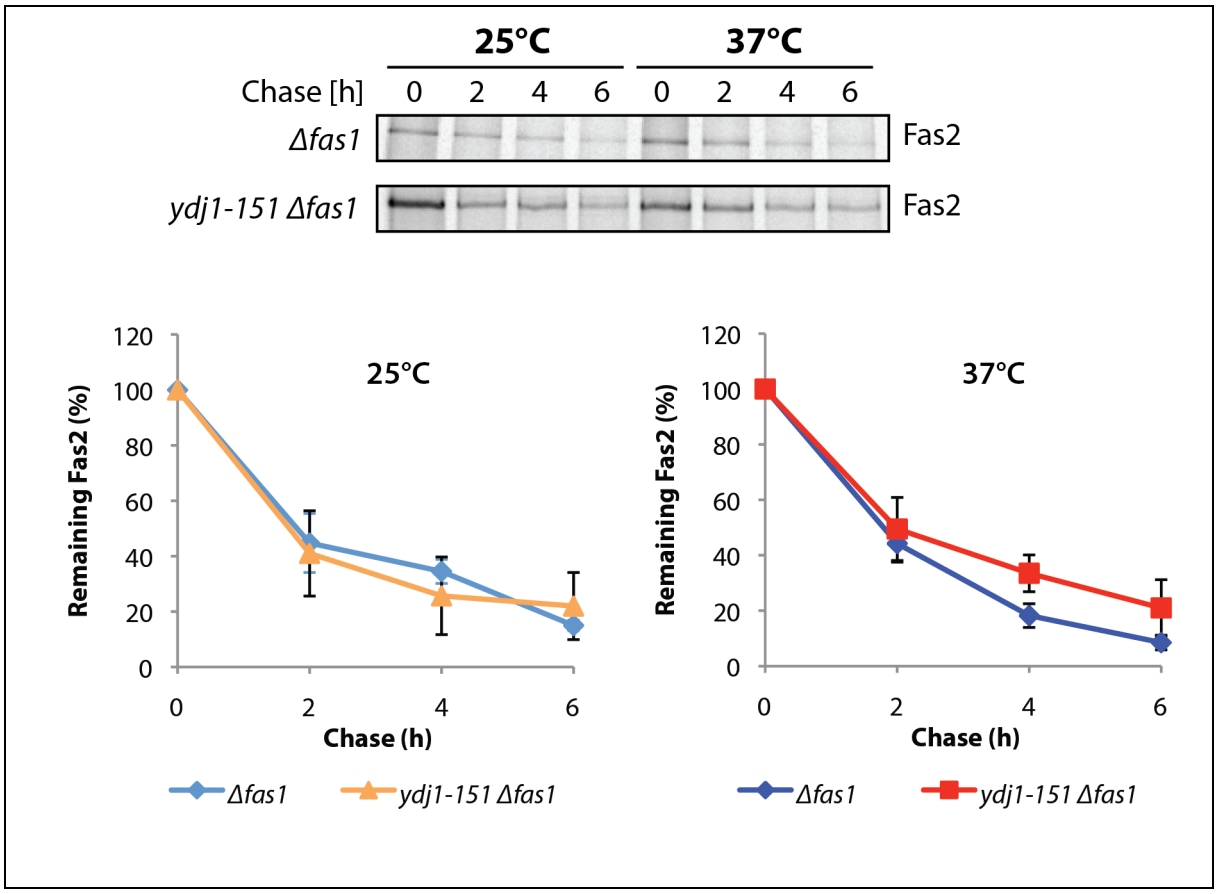

Figure 10. **The temperature sensitive *ydj1-151* allele has no effect on orphan Fas2 degradation.** Pulse chase analyses of orphan Fas2 in *Δfas1* (YMS 5) and *ydj1-151 Δfas1* (YMS 158) cells were performed according to the protocol for temperature sensitive mutants described in chapter 4.4.6. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean.

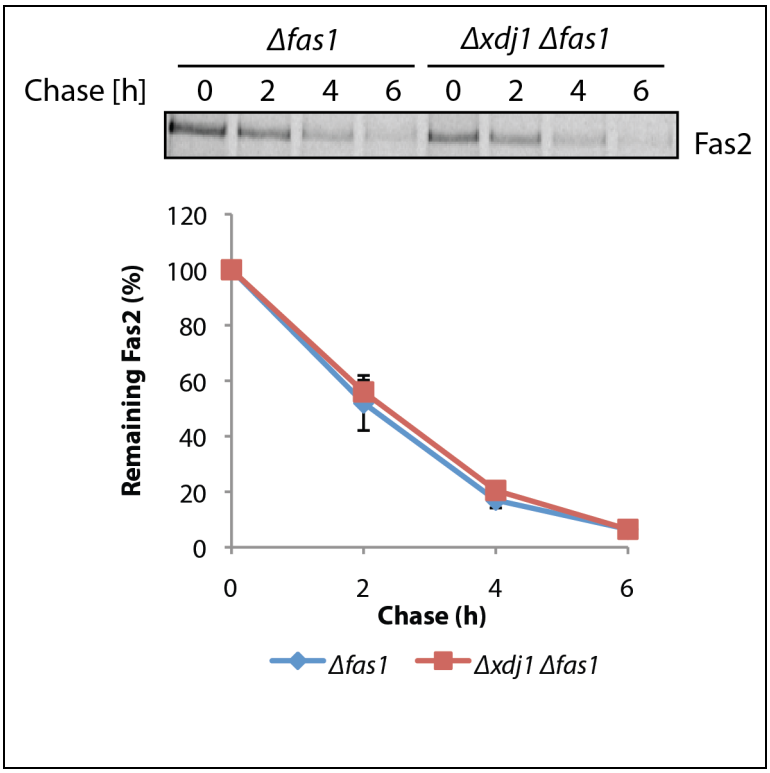

Figure 11. **Deletion of the Hsp40 chaperone Xdj1 has no influence on orphan Fas2 degradation.** Pulse chase analyses of orphan Fas2 in *Δfas1* (YMS 5)- and *Δxdj1 Δfas1* (YMS 152) cells were performed, according to the protocol described in chapter 4.4.6. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of two independent experiments. The error bars represent the standard deviation of the mean.

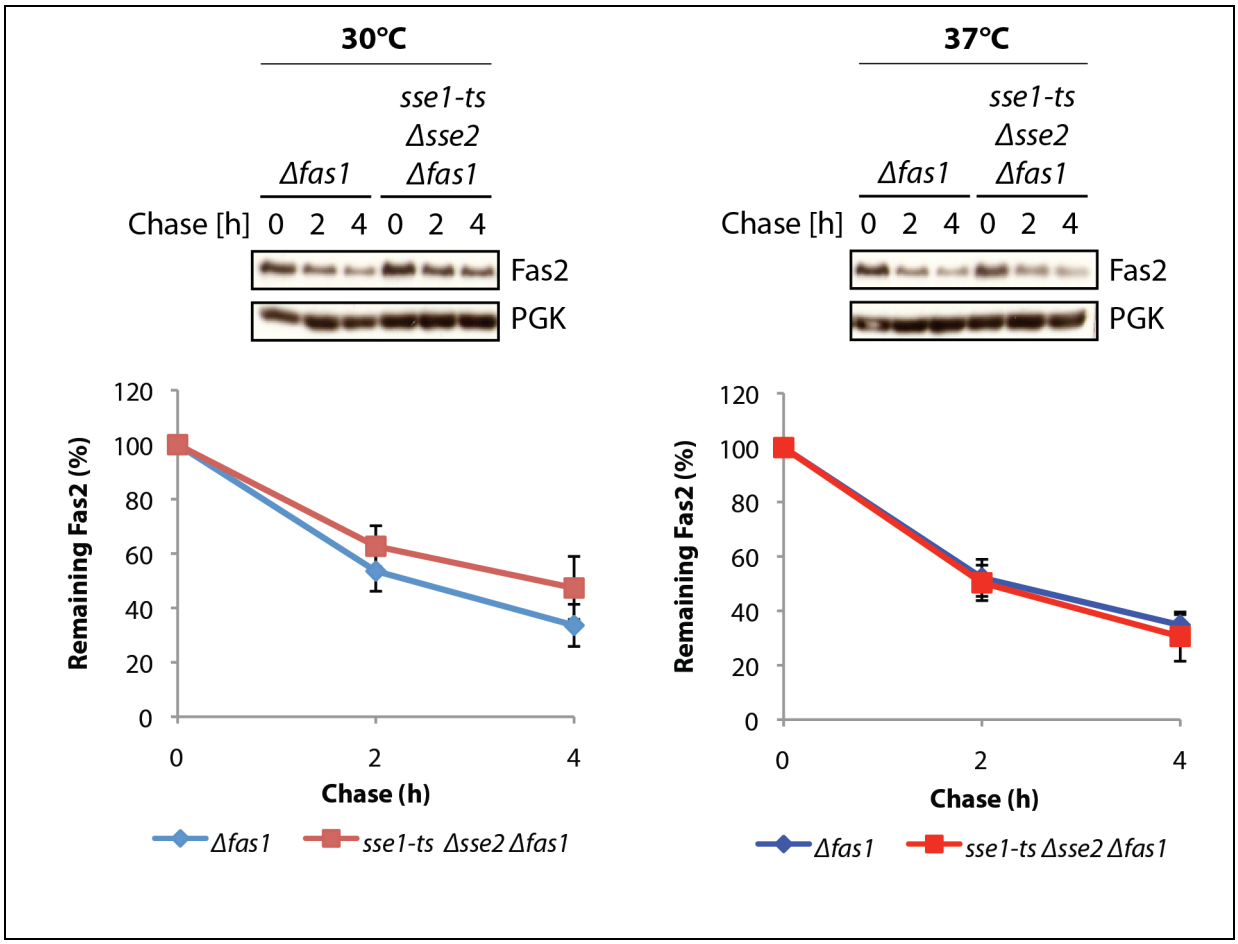

Figure 12. **The Hsp110 chaperones Sse1 and Sse2 are not required for orphan Fas2 degradation.** Cycloheximide chase experiments of orphan Fas2 in *Δfas1* (YMS 50)- and the *sse1^{ts} Δsse2 Δfas1* (YMS 63) cells were performed. Briefly, all strains were grown in YPD-FA media at 30°C until they reached the exponential growth phase. Cycloheximide was added one hour after incubation at 30°C or 37°C. Samples were collected at the indicated time points. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control. Band intensities of Fas2 were quantified with ImageJ and plotted as graph using Excel. Plotted data represent the mean values of two independent experiments. Error bars represent the standard deviation of the mean.

5.2.3 Inhibition of Hsp90 chaperones by geldanamycin has no effect on orphan Fas2 degradation

Due to a large-scale protein-protein interaction study (Krogan et al, 2006), which revealed a interaction of Fas2-TAP and Hsp82 in yeast wild type cells it can hypothesized that the moderate long *in vivo* half-life of orphan Fas2 ($t_{1/2}$ about 2h) of this natural, non-mutated protein could be due to possible interaction with Hsp90 chaperones (Hsc82 and Hsp82). Several protein-folding intermediates need the assistance of the Hsp90 activity to fold properly. Therefore, it is proposed that an Hsp90 chaperone functions as a holdase, which is defined as binding of a protein folding intermediate to prevent non-specific (self)-aggregation (Beissinger & Buchner, 1998). Recent studies also suggest that Hsp90 and several cochaperones function in the assembly of protein complexes (Bansal et al, 2004; Imai et al, 2003) and protein degradation (Arndt et al, 2007; McClellan et al, 2005; Nillegoda et al, 2010). Hence, it was of high interest whether the Hsp90 chaperones are involved in the protein quality control of orphan Fas2. It is well established that geldanamycin (GA) is a potent competitive inhibitor of the Hsp90 chaperones. Before measuring the degradation rate of orphan Fas2 in the presence of GA, a *PDR5* deletion was introduced into the *Δfas1* mutant strain. Pdr5 is a multidrug transporter in the plasma membrane that mediates resistance to many xenobiotic compounds (e.g. geldanamycin) by transporting them outside of the cell. The degradation of orphan Fas2 in a *Δpdr5 Δfas1* strain treated with 50 μM GA (solved in DMSO) was monitored in a pulse chase experiment. As shown in Figure 13, inhibition of the Hsp90 chaperones with GA has no effect on the degradation of orphan Fas2. The *in vivo* half-life of the orphan substrate is the same in GA-treated (GA and DMSO) and GA-untreated (only DMSO) cells the same ($t_{1/2}$=2h). Thus, Hsp90 chaperones seem to play no important role in the degradation of orphan Fas2.

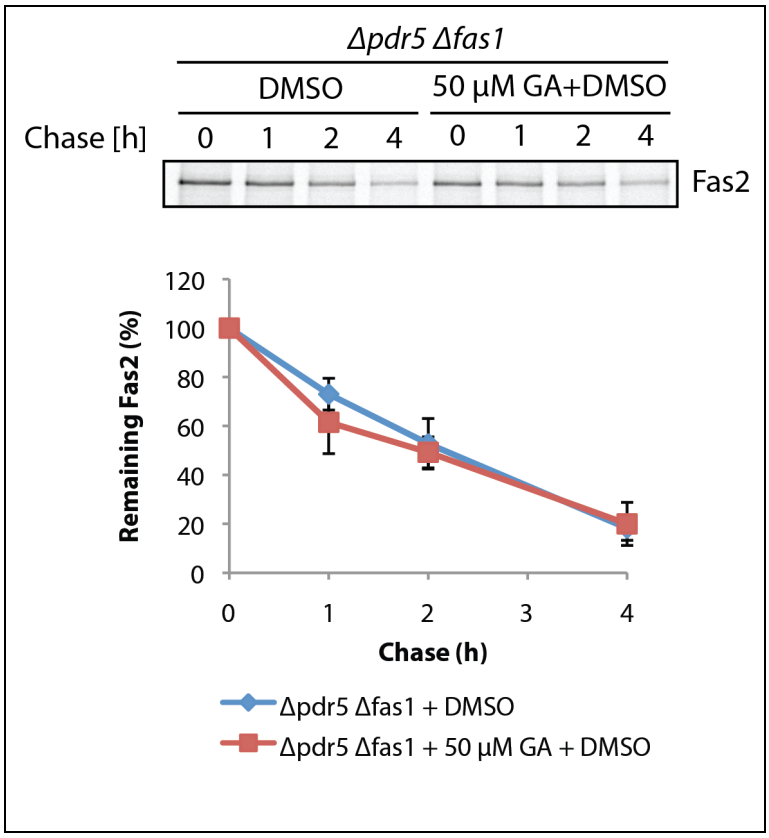

Figure 13. **Inhibition of the Hsp90 chaperone activity by geldanamycin has no influence on orphan Fas2 degradation.** Pulse chase analyses of orphan Fas2 in a *Δpdr5 Δfas1* (YMS 87) deletion strain were performed, treated with Geldanamycin (solved in DMSO) and, only with DMSO (negative control) 30 min before the chase phase. GA was added to give a final concentration of 50 µM. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and ImageQuant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean. GA = Geldanamycin, DMSO = Dimethylsulfoxid.

5.2.4 Degradation of orphan Fas2 is not disturbed in the absence of Sti1

As the Hsp70 cochaperone Ydj1 and others (see chapter 5.2.3) have no effect on orphan Fas2 degradation I tested the influence of the chaperone Sti1. Besides its well-described role as Hsp90 cochaperone, it has been found that Sti1 possesses the ability to stimulate Hsp70 ATPase activity (Wegele et al, 2003). In addition, a previous study had found an involvement of Sti1 in the degradation of the heterologously expressed orphan protein VHL in yeast (McClellan et al, 2005). In a pulse chase experiment the proteolytic fate of orphan Fas2 was followed in a *Δsti1 Δfas1* strain. No significant difference in the degradation rate of Fas2 compared to the control strain *Δfas1* was observed (Figure 14). Consequently, Sti1 is not required for the degradation of orphan Fas2.

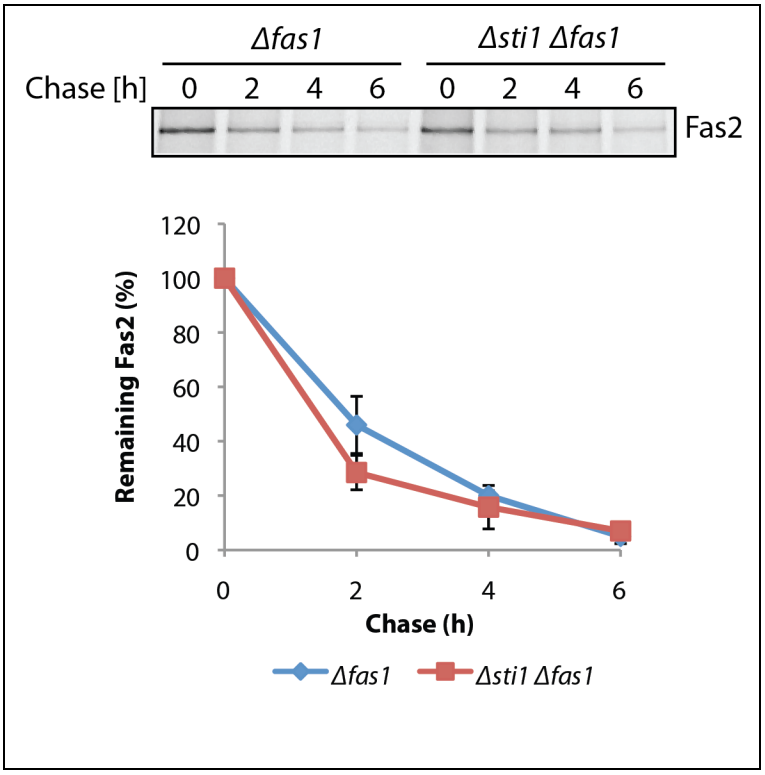

Figure 14. **Orphan Fas2 degradation is not dependent on the chaperone Sti1.** Pulse chase analyses of orphan Fas2 in *Δfas1* (YMS 5)- and a *Δsti1 Δfas1* (YMS 91) cells were performed, according to the protocol described in chapter 4.4.6. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of four independent experiments. The error bars represent the standard deviation of the mean.

5.2.5 Neither Hsp104 nor Hsp26 and Hsp42 are required for orphan Fas2 degradation

So far, only the Hsp70 chaperone Ssa1 has been identified important for the protein quality control of orphan Fas2 (see chapter 5.2.1). The question raised whether additional chaperones, including Hsp104 as well as Hsp26 and Hsp42 might be involved in the degradation process of the orphan Fas2 substrate. The only member of the Hsp100 family, Hsp104, is a cytosolic chaperone that works together with Ssa1 and Ydj1 in order to disassemble protein aggregates.

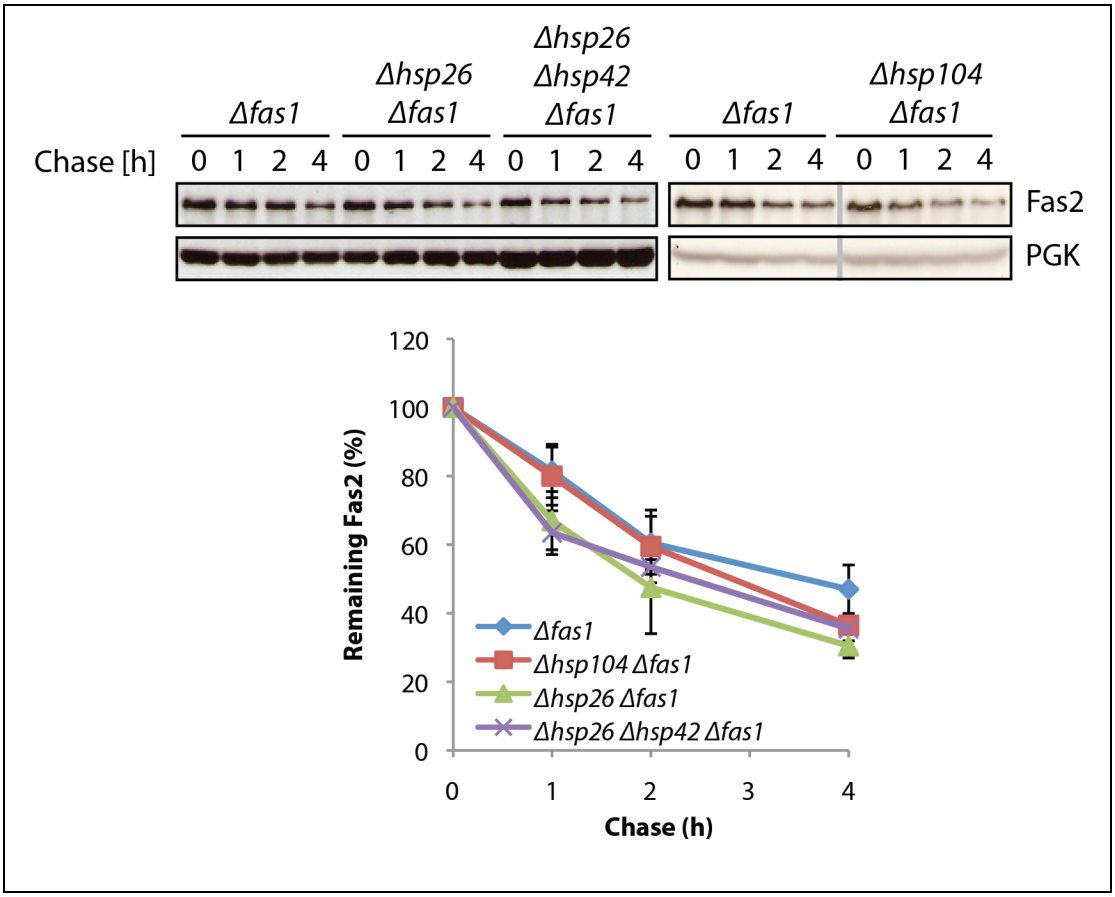

Figure 15. **The Hsp104 chaperone and the small heat shock proteins, Hsp26 and Hsp42, are not involved in the degradation of orphan Fas2.** Cycloheximide chase experiments of orphan Fas2 in *Δfas1* (YMS 5)-, *Δhsp104 Δfas1* (YMS 185)-, *Δhsp26 Δfas1* (YMS 42)- and *Δhsp26 Δhsp42 Δfas1* (YMS 43) cells were performed. Cycloheximide was added (t=0h) to exponentially grown cells, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control. Plotted data represent the mean values of two independent experiments. The error bars represent the standard deviation of the mean.

Additional lanes in the immunoblot were removed, in which another mutant was shown. This is indicated with a grey vertical line.

The small heat shock proteins Hsp26 and Hsp42 interact under stress conditions with unfolded or partially folded proteins to prevent their aggregation. Already aggregated substrates can be kept in a barrel-shaped oligomer "depot", consisting of Hsp26- and Hsp42 proteins, where they eventually undergo refolding in the presence of the protein refolding machinery Hsp104/Ssa1/Ydj1 (Cashikar et al, 2005; Haslbeck et al, 2005). Using cycloheximide chase experiments, I followed the degradation of orphan Fas2 in an *Δhsp104 Δfas1-*, *Δhsp26 Δfas1-* and in an *Δhsp26 Δhsp42 Δfas1* mutant strain. As shown in Figure 15 the degradation behaviour of orphan Fas2 is similar in all tested chaperone mutants compared to the control strain *Δfas1*, indicating that these factors are not required for the Ssa1 mediated degradation of orphan Fas2.

5.3 The role of the ubiquitin proteasome system in the protein quality control of orphan Fas2

With a few exceptions (Asher et al, 2006; Shringarpure et al, 2003; Zhang et al, 2003) a protein must be tagged with a polyubiquitin chain (preferably K48-linked Ub-chain) and present an unfolded peptide sequence of at least 20-25 amino acids ("loose end") to become a target of the 26S proteasome (Prakash et al, 2004; Wolf & Hilt, 2004). (Poly)-ubiquitination is executed by the sequential action of three different enzymes called E1, E2 and E3 (see chapter 3.2.2 and 3.2.2.1). As orphan Fas2 is a substrate of the 26S proteasome (see Figure 5) and (Egner et al, 1993), it was of great interest to elucidate which E2 and E3 enzyme(s) participate to the polyubiquitination process, and thus target Fas2 to the proteasomal degradation process.

5.3.1 The absence of the two cytosolic E2 enzymes, Ubc2 and Ubc4, strongly delays proteasome mediated degradation of orphan Fas2

As orphan Fas2 is a cytosolic protein (see Figure 6), the influence of cytosolically located yeast E2 enzymes (Ubc2, Ubc4 and Ubc8) on orphan Fas2 quality control were analyzed. Several studies have been reported that the two cytosolic ubiquitin-conjugating enzymes, Ubc4 and Ubc5, are involved in E3-mediated polyubiquitination of several cytosolic misfolded proteins (Medicherla & Goldberg, 2008; Park et al, 2007; Seufert & Jentsch, 1990). As the attempt failed to generate a *FAS1* gene deletion in a *Δubc4 Δubc5* double deletion, it was not possible to monitor the degradation of orphan Fas2 in the absence of Ubc4 and Ubc5. It was also not possible to generate this genotype in the W303 background. Despite several attempts, it was not even possible to generate a *UBC4 UBC5* double deletion, suggesting these mutations might be lethal in this strain background W303. Therefore the influence of single *UBC* deletions (*Δubc2*, *Δubc4*, *Δubc8*) on proteasome-mediated turnover of orphan Fas2 were tested in strains each deleted in *FAS1*. No significant effects could be observed (Maxi Kanold, Diplomarbeit, 2010). However, pulse chase analyses revealed that, in contrast to the single deletion of *UBC2* and *UBC4*, the combined lack of both E2 enzymes has a strong influence on the degradation of orphan Fas2 (Figure 16). This strongly indicates a complementing function of Ubc2 and Ubc4 regarding to the quality control process of orphan Fas2. As

degradation is not completely blocked in the *Δubc2 Δubc4 Δfas1* mutant strain additional E2(s) might be involved in orphan Fas2 turnover.

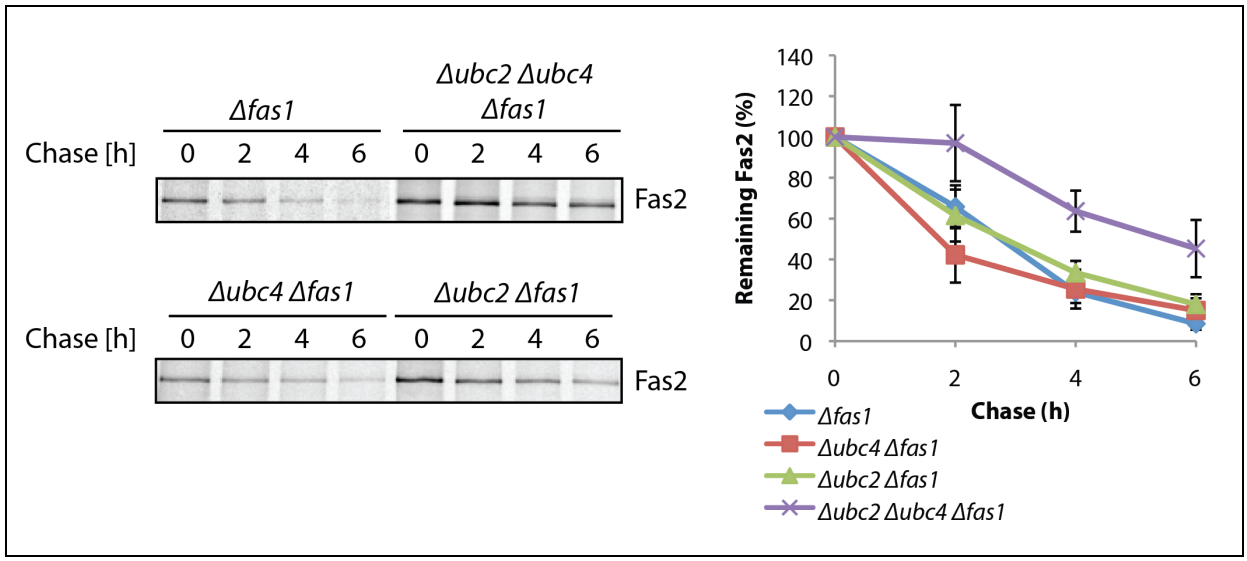

Figure 16. **Simultaneous lack of two E2-enzymes, Ubc2 and Ubc4, impairs orphan Fas2 degradation.** Pulse chase analyses of orphan Fas2 with the deletions strains: *Δfas1* (YMS 5), *Δubc2 Δfas1* (YMS 119), *Δubc4 Δfas1* (YMS 155) and *Δubc2 Δubc4 Δfas1* (YMS 154) were performed according to the protocol described in chapter 4.4.6. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean.

5.3.2 The E3 RING ligase Ubr1 is required for orphan Fas2 degradation

As orphan Fas2 is localized to the cytosol (see Figure 6), I tested the effect of different cytosolic E3 ligases on the degradation of orphan Fas2 (*Δfas1*) in respective E3 deletion strains by using cycloheximide- and pulse chase analysis. As seen in Figure 17, deletion of the gene encoding the ubiquitin ligase Ubr1 leads to a considerable stabilization of orphan Fas2, indicating involvement of Ubr1 in the degradation of this cytosolic orphan protein. As for the *Δubc2 Δubc4 Δfas1* mutant (see Figure 16), the *in vivo* half-life of orphan Fas2 is increased from 2 hours (*Δfas1*) to 6 hours (*Δubr1 Δfas1*). As degradation is not completely blocked other E3 ligase(s) might be involved in ubiquitination of orphan Fas2. Compared to orphan Fas2 an accelerated degradation of orphan Fas2-EGFP had been observed (see Figure 7). Therefore the question was addressed whether

degradation of this epitope-tagged protein is also dependent on Ubr1. Compared to the *Δfas1* deletion strain the disappearance of orphan Fas2-EGFP is also delayed in the absence of the E3 RING ligase Ubr1, indicating that the EGFP epitope has no qualitative effect on Ubr1 mediated degradation of the orphan protein.

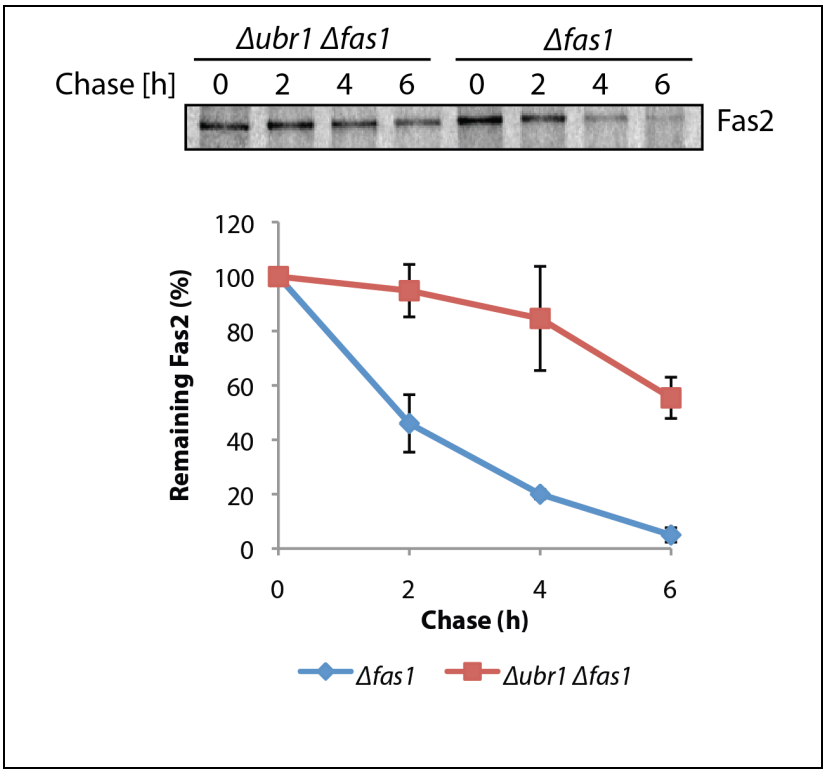

Figure 17. **Orphan Fas2 degradation requires the E3 RING ligase Ubr1.** Pulse chase analyses of orphan Fas2 in *Δfas1* (YMS 5) – and *Δubr1 Δfas1* (YMS 23) cells were performed according to the protocol described in chapter 4.4.6. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and ImageQuant (Amersham Bioscience). Plotted data represent the mean values of four independent experiments. The error bars represent the standard deviation of the mean.

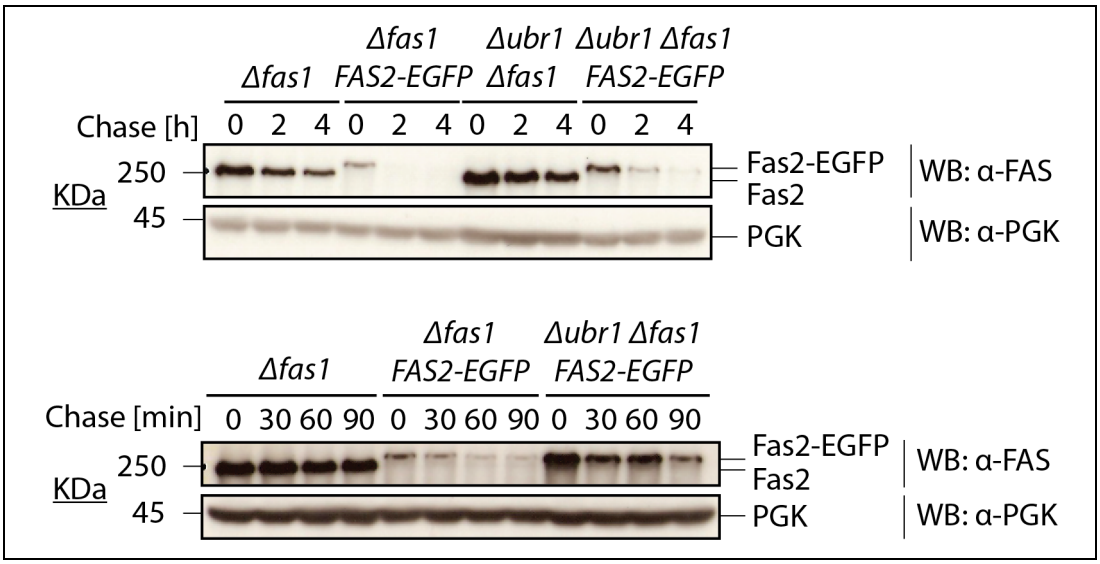

Figure 18. **Ubr1 is also involved in degradation of C-terminally EGFP tagged orphan Fas2.** Cycloheximide chase analyses of endogenously expressed orphan Fas2 in *Δfas1* (YMS 5) and *Δubr1 Δfas1* (YMS 23) cells as well as endogenously expressed orphan Fas2-EGFP in *Δfas1* (YMS 131) and *Δubr1 Δfas1* (YMS 132) cells were performed. Cycloheximide was added (t=0h) to exponentially grown cells, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control.

5.3.3 A point mutation in the type-1 binding site, but not in the type-2 binding site of Ubr1, strongly delays the degradation of orphan Fas2

It is known that the so-called type-1- and type-2 substrate binding sites of E3 ligase Ubr1 are necessary for degradation of type-1- and type-2 N-end rule substrates, respectively. The Ubr1 type-1- and the Ubr1 type-2 binding site specifically recognizes basic N-terminal amino acid residues and bulky hydrophobic N-terminal amino acid residues, respectively. The N-terminal amino acid sequence of Fas2 is Met-Lys-Pro-Glu- (www.yeastgenome.org). According to the "Sherman-rule", methionine at the N-terminus can be only cleaved off when the side chain of the second amino acid has a radius of gyration of 1.29 Å or less (Moerschell et al, 1990; Sherman et al, 1985). The side chain of lysine, which is the second amino acid at the N-terminus of Fas2, has a radius of gyration of 2.08 Å. Thus, according to the "Sherman rule" the N-terminal methionine of Fas2 cannot be cleaved. However, it cannot be excluded that the amino terminus of orphan Fas2 is posttranslational processed, e.g. by endopeptidase(s), generating a destabilizing N-end rule amino terminus recognized by Ubr1. In order to prove whether

these substrate binding sites of Ubr1 play a role in the degradation of orphan Fas2 I applied pulse chase analysis of orphan Fas2 in an *Δubr1 Δfas1* strain overexpressing either Ubr1-HA, Ubr1^{D176E}-HA (type-1 mutant), Ubr1^{P406S}-HA (type-2 mutant) from a high copy plasmid, or containing an empty vector. As shown in Figure 19 orphan Fas2 degradation is strongly delayed in a similar manner in the *Δubr1 Δfas1* strain carrying the empty vector (black line) or the plasmid expressing Ubr1^{D176E}-HA (red line) and is restored when Ubr1-HA (green line) or Ubr1^{P406S}-HA (blue line) is expressed at the same time. This indicates the need of a functional type-1 binding site for Ubr1-mediated degradation of orphan Fas2. As the rate of degradation of orphan Fas2 is almost the same in the *Δubr1 Δfas1* deletion strain expressing Ubr1-HA or Flag-Ubr1^{P406S}-HA, the type-2 binding site of Ubr1 seems to play no important role in the Ubr1-dependent degradation of orphan Fas2.

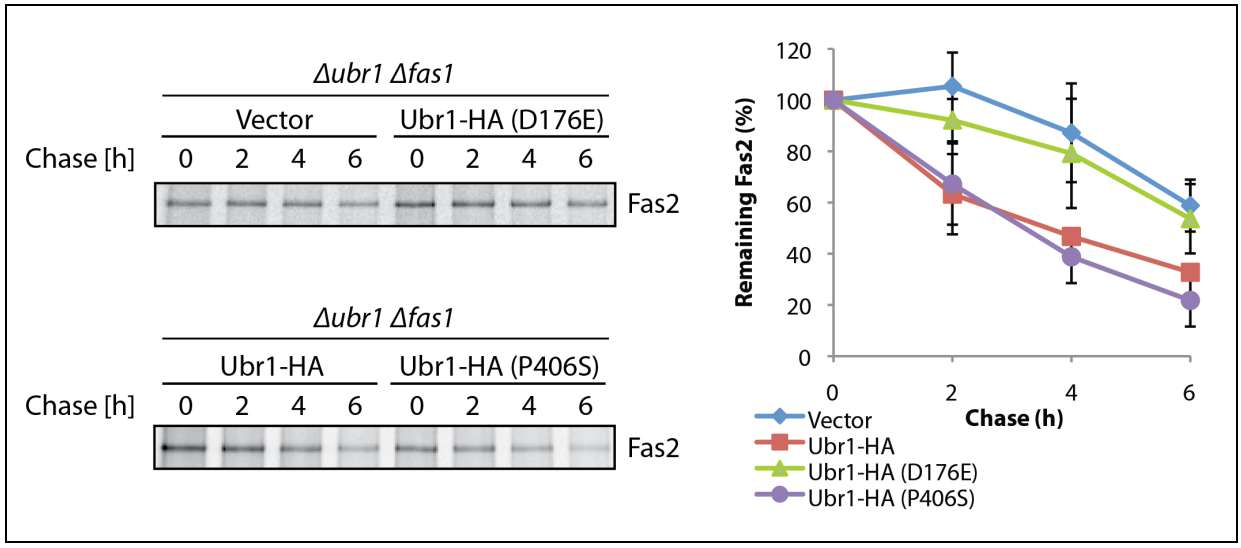

Figure 19. **Degradation of orphan Fas2 is strongly delayed in a *Δubr1 Δfas1* strain expressing an Ubr1 mutant protein, defective in degradation of type-1 N-end rule substrates.** Pulse chase analyses of orphan Fas2 were performed in *Δubr1 Δfas1* (YMS 23) cells, expressing from a high copy plasmid either HA-tagged wild type Ubr1 (Ubr1-HA, red line) or HA-tagged Ubr1 point mutants, defective in degradation of type-1 (Ubr1^{D176E}-HA, green line) or type-2 (Ubr1^{P406S}-HA, purple line) N-end rule substrates, or containing empty vector (blue line) as negative control. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean.

5.3.4 Orphan Fas2 degradation requires the Ubr1 RING domain

As I identified the E3 RING ligase Ubr1 necessary for proteasomal degradation of orphan Fas2, it was of great interest to look whether the RING domain of Ubr1 plays an essential role in this degradation process. The RING domain of a RING E3 catalyzes the covalent attachment of an ubiquitin molecule onto an E3 client substrate. Here, the RING E3 binds a single ubiquitin molecule, received from an ubiquitin conjugating enzyme (E2), and transfers it onto a lysine residue of a substrate or onto an ubiquitin chain, which is already attached to the substrate. Subsequently, the formed polyubiquitin chain(s) can serve(s) as a signal to target the substrate for proteasomal degradation.

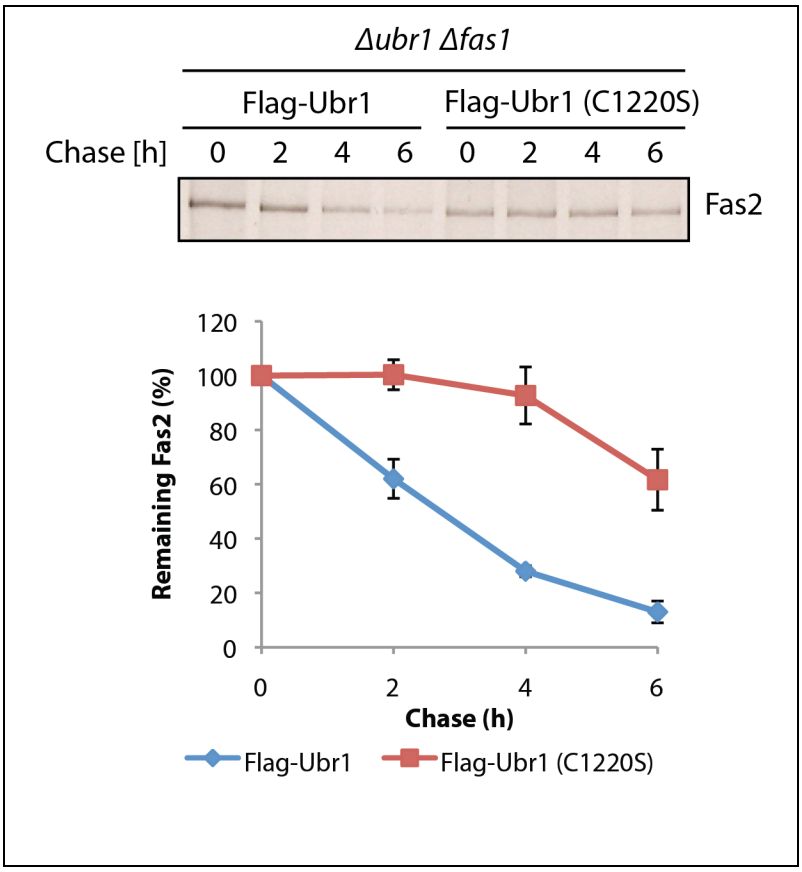

Figure 20. **The RING domain of Ubr1 is important for orphan Fas2 degradation.** Pulse chase analyses of orphan Fas2 in *Δubr1 Δfas1* cells were performed expressing either Flag tagged Ubr1 (Flag-Ubr1, blue line) or an inactive RING mutant of Ubr1 (Flag-Ubr1^{C1220S}, red line) from a high copy plasmid. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean.

To test whether the RING domain of Ubr1 is needed for removal of orphan Fas2 a high copy plasmid encoding a Flag-tagged catalytically inactive mutant of Ubr1 (Flag-Ubr1^{C1220S}) was transformed into a *Δubr1 Δfas1* deletion strain and the proteolytical fate of the orphan substrate by using pulse chase analysis has been monitored. A plasmid expressing functional Ubr1 (Flag-Ubr1) transformed into same strain background served as negative control. As shown in Figure 20, expression of Flag-Ubr1 in an *Δubr1 Δfas1* deletion strain re-establishes degradation of orphan Fas2. The presence of an inactive RING mutant of Ubr1 (FlagUbr1^{C1220S}) in the same strain background prevents proteasome-mediated degradation of Fas2. As degradation of the orphan substrate requires a functional RING domain of Ubr1, it can be assumed that this E3 ligase is responsible for the polyubiquitination of orphan Fas2.

5.3.5 Physical interaction between orphan Fas2 and Ubr1

Due to the requirement of Ubr1, its RING domain as well as the type-1 substrate-binding site, for degradation of orphan Fas2 it is very likely that the E3 ligase physically interacts with this orphan protein. As the FAS antibody is not suitable to perform immunoprecipitation of native Fas2 I tagged chromosomal *FAS2* gene with the TAP epitope, allowing me to pull down the orphan Fas2 protein by IgG Sepharose™ beads. To prove whether the chromosomally expressed Fas2-TAP fusion protein is still functional in fatty acid synthesis, growth tests were performed as described in chapter 5.1.2. It turned out that the TAP epitope of Fas2 has no influence, on proper fatty acid synthesis and on cell division time (data not shown). To test whether the TAP epitope of orphan Fas2-TAP impairs the degradation of Fas2 in a *Δfas1*- and in a *Δubr1 Δfas1* deletion mutant cycloheximide chase experiments with respective control strains were performed. When compared with untagged orphan Fas2, the C-terminally fused TAP epitope slightly increases the turnover rate of orphan Fas2-TAP in a *Δfas1* strain (Figure 21). Like for untagged orphan Fas2 the turnover of orphan Fas2-TAP is still dependent on the E3 ligase Ubr1 (Figure 21). Thus, it seems to be that the C-terminal TAP epitope of orphan Fas2-TAP decreases slightly decreases the *in vivo* half-life of the fusion protein, but does not affect its Ubr1-dependent degradation.

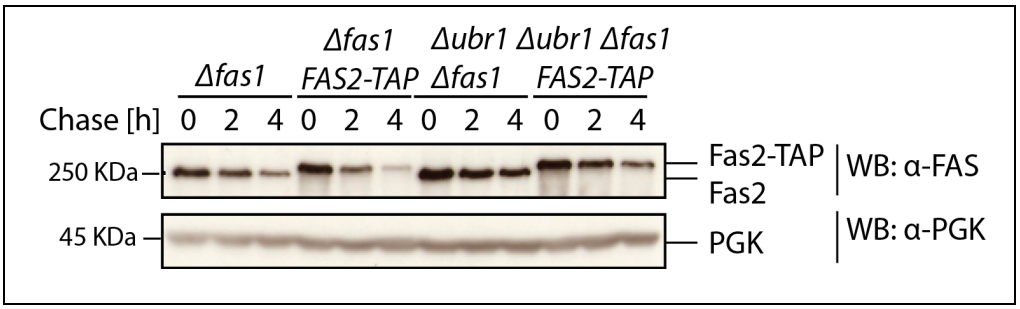

Figure 21. **Orphan Fas2-TAP is slightly faster degraded than orphan Fas2.** Cycloheximide chase analyses of endogenously expressed orphan Fas2 in *Δfas1* (YMS 5) and *Δubr1 Δfas1* (YMS 23) cells as well as endogenously expressed orphan Fas2-TAP in *Δfas1* (YMS 51) and *Δubr1 Δfas1* (YMS 64) cells were performed. Cycloheximide was added (t=0h) to exponentially grown cells, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- or PGK antibody, respectively. PGK served as loading control.

To verify a possible interaction between orphan Fas2 and Ubr1 TAP pull down experiments with an *Δubr1 Δfas1* deletion strain, expressing endogenous TAP-tagged Fas2 and carrying a high-copy plasmid encoding either HA-tagged Ubr1 (Ubr1-HA) or the corresponding empty vector (negative control) were performed. In addition a further control experiment to was done determining whether Ubr1-HA or untagged Fas2 bind unspecifically to IgG Sepharose beads, thereby using an *Δubr1 Δfas1* strain expressing endogenous Fas2, and, from a high-copy plasmid, Ubr1-HA.

After lysis of yeast cells under native condition (see chapter 4.4.2) Fas2-TAP was captured from cell extracts with IgG beads (see chapter 4.4.8) and then released either by boiling in urea loading buffer (Figure 22A) or by proteolytical cleavage with TEV protease (Figure 22B). As shown in Figure 22A (Lane 5) and Figure 22B (Lane 6), Ubr1-HA was co-purified with orphan Fas2-TAP, indicating that orphan Fas2 and the E3 ligase Ubr1 interact *in vivo*. As control, 3-phosphoglycerate-kinase was not found in the purified Fas2-TAP sample, suggesting that the interaction between Fas2 and Ubr1 is specific. To exclude that the co-purification of Ubr1-HA is due to an unspecific binding of Ubr1-HA to the IgG Sepharose beads I performed a pull down assay with the same strain background but untagged Fas2 (*Δubr1 Δfas1* deletion strain, expressing endogenous Fas2 and, from a plasmid, Ubr1-HA). In this control only very small amount of Ubr1-HA was detected in the TAP pull down fraction (Figure 22A, Lane 6).

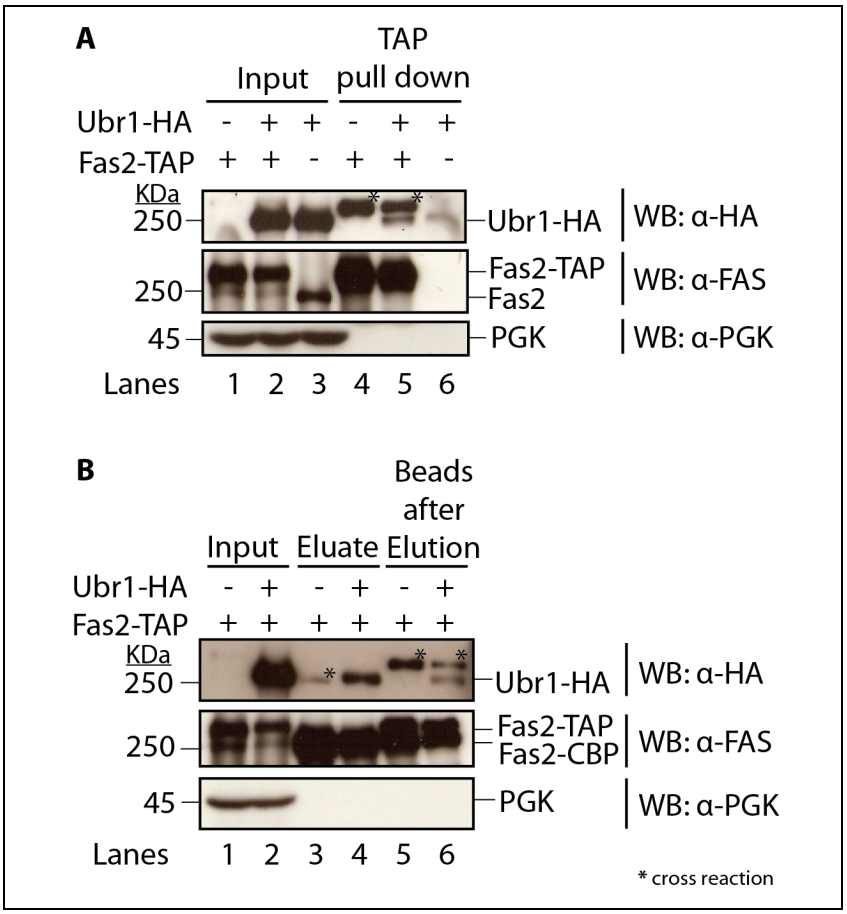

Figure 22. **Physical interaction between orphan Fas2 and Ubr1.** High copy plasmid expressing HA-tagged wild type Ubr1 under the control of the *ADH1* promoter and the respective empty vector (negative control) were separately transformed into a *Δubr1 Δfas1 FAS2-TAP* (YMS 64) strain. The *Δubr1 Δfas1 FAS2* (YMS 63) strain containing the same Ubr1-HA plasmid served as control to detect unspecific binding of Ubr1-HA and untagged Fas2 to IgG Sepharose beads (A: Lane 6). Supernatant obtained by native cell lysis and subsequent centrifugation at 16.200 g for 20 min was used as input (A: Lanes 1-3, B: Lanes 1 and 2). From the supernatant, Fas2-TAP was purified with IgG Sepharose beads and released either by direct boiling in urea loading buffer (A: Lane 4 and 5) or by cleavage with TEV protease (B: Lanes 3 and 4). After TEV-mediated cleavage of Fas2-TAP IgG beads were boiled in urea loading buffer to determine cleavage efficiency of Fas2-TAP (B: Lane 5 and 6). All samples were subjected to SDS-PAGE followed by immunoblotting using HA-, FAS- and PGK antibodies.

The TEV protease mediated cleavage of IgG-bound Fas2-TAP has the advantage to avoid the appearance of an additional band on the Western blot (Figure 22A, marked by *) that runs unfortunately almost at the same height (~250 KDa) like Ubr1-HA. During TEV cleavage of Fas2-TAP the ProteinA-module of the TAP epitope remains still attached to the IgG beads whereas the generated Fas2-CBP is eluted into the aqueous

phase. When analyzing the eluate on a Western blot the additional band disappears (compare Figure 22A, Lanes 4,5 and Figure 22B, Lanes 3,4). One explanation for the appearance of the cross-reaction could be that the constant region of the secondary antibody binds to the ProteinA-module of the TAP epitope. After the elution step, IgG Sepharose beads were boiled in urea loading buffer to check cleavage efficiency of the TEV protease under the conditions described in chapter 4.4.8. As can be seen in Figure 22B (Lane 5 and 6) there is still some material left on the beads, indicating that conditions for TEV protease mediated cleavage of Fas2-TAP can still be improved. Nevertheless, the conditions used in our experiment were sufficient to show that orphan Fas2 and Ubr1 are interactors *in vivo*.

5.3.6 An Ubr1 mutant, defective in degradation of type-1 or type-2 N-end rule substrates, or carrying a catalytically inactive RING domain, is still able to bind orphan Fas2

Proteasomal degradation of classical type-1- and type-2 N-end rule substrates (e.g. Arg-β-Gal and Leu-β-Gal, respectively) occurs via binding to the corresponding type-1 or type-2 substrate binding site of Ubr1, followed by Ubr1-mediated polyubiquitination. Inactivation of these substrate-binding sites (e.g. by a point mutation) abolish the ability of Ubr1 to degrade the corresponding N-end rule substrates (Xia et al, 2008). As I could show that Ubr1-depdendent degradation of orphan Fas2 is strongly delayed when Ubr1 carries a point mutation in the type-1 substrate-binding site (D176E) or in the RING domain (C1220S) (see chapter 5.3.3 and 5.3.4) it raised the question whether these mutants, and here especially the type-1 mutant, are still able to bind the orphan protein. To address this question the corresponding *UBR1*-expressing plasmids were transformed into separate *Δubr1 Δfas1 FAS2-TAP* strains and tested whether the different Ubr1 mutant proteins could be co-purified with orphan Fas2-TAP. Here, Fas2-TAP was eluated from the IgG beads by cleavage with TEV protease (see also chapter 5.3.5). As is clearly shown in Figure 23A, HA-tagged Ubr1^{D176E} (Lane 8) and HA-tagged Ubr1^{P406S} (Lane 9) as well as Flag-tagged Ubr1^{C1220S} (Figure 23B, Lane 6) could be co-purified after Fas2-TAP pull down. This indicates that the *in vivo* interaction between Ubr1 and orphan Fas2 is not dependent on functional type-1- or type-2-substrate binding sites and a functional RING domain of Ubr1.

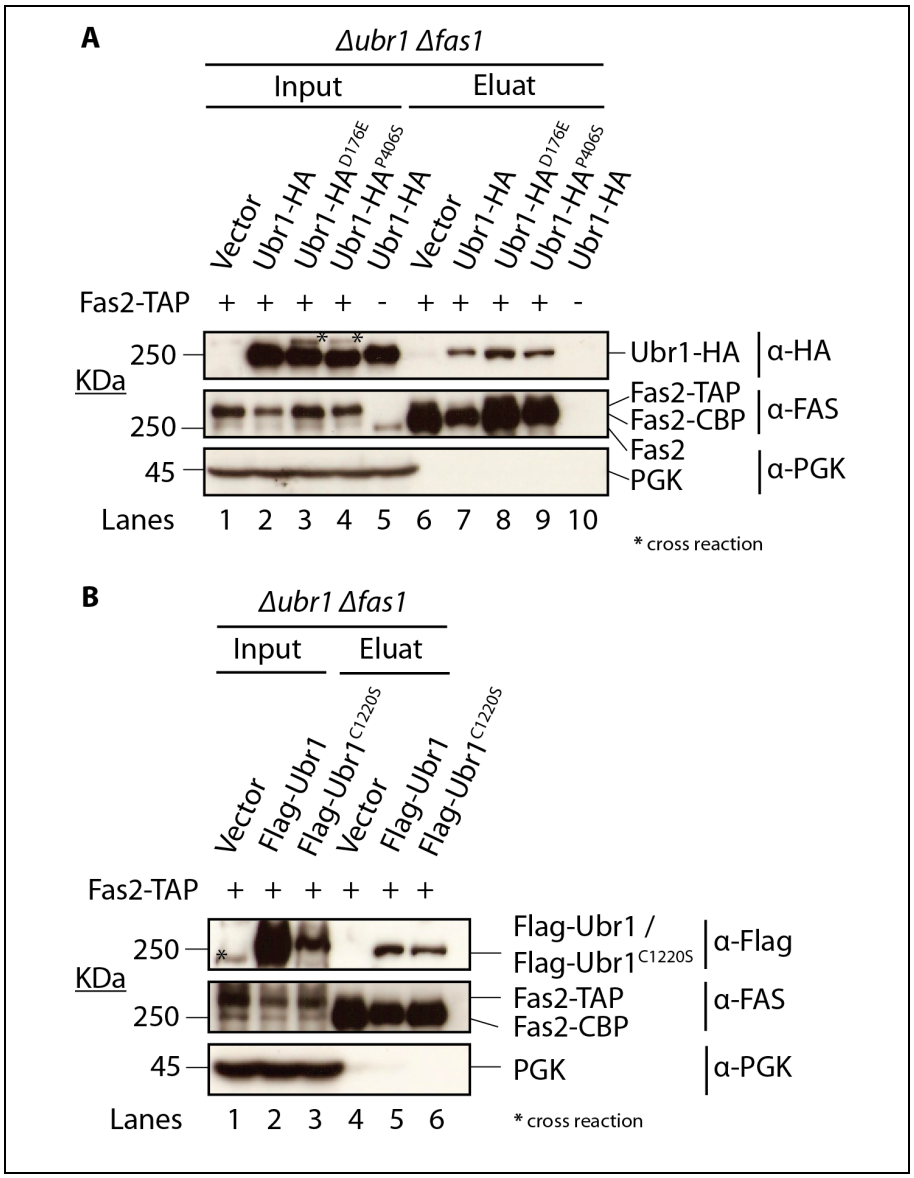

Figure 23. **Interaction between orphan Fas2 and Ubr1 depends neither on functional type-1-, type-2-substrate binding sites nor on a functional RING domain of Ubr1. A:** High copy plasmid expressing either HA-tagged wild type Ubr1 (Ubr1-HA) or mutated Ubr1 (Ubr1^{D176E}-HA, Ubr1^{P406S}-HA) under the control of the *ADH1* promoter and the corresponding empty vector (negative control) were separately transformed into a *Δubr1 Δfas1 FAS2-TAP* (YMS 64) strain. The *Δubr1 Δfas1 FAS2* (YMS 63) strain containing the same Ubr1-HA plasmid served as control to detect unspecific binding of Ubr1-HA and untagged Fas2 to IgG Sepharose beads (Lane 10). Supernatant obtained by native cell lysis and subsequent centrifugation at 16.200 g for 20 min was used as input (Lanes 1-5). From the supernatant, Fas2-TAP was purified with IgG Sepharose beads and released by cleavage with TEV protease (Lanes 6-9). All samples were subjected to SDS-PAGE followed by immunoblotting using HA-, FAS- and PGK antibodies. **B:** like for A, but performed with plasmids expressing Flag-tagged wild type Ubr1 (Flag-Ubr1) or a Flag-tagged Ubr1 RING mutant (Flag-Ubr1^{C1220S}). Immunoblot was incubated with Flag-, FAS- and PGK antibodies.

Fas2-TAP mediated co-purification of HA-tagged wild type Ubr1 (Figure 23A, Lane 7), Flag-tagged wild type Ubr1 (Figure 23B, Lane 5) and 3-phosphoglycerate-kinase (Figure 23A, Lane 6-10 and Figure 23B, Lane 4-6) served as positive- and negative interaction controls, respectively. Whereas the two different epitope-tagged versions of wild type Ubr1 (Ubr1-HA or Flag-Ubr1) were found in the purified Fas2-TAP sample, 3-phosphoglycerate-kinase was not, suggesting that the *in vivo* interaction between orphan Fas2 and the different Ubr1 mutants (Ubr1^{D176E}-HA, Ubr1^{P406S}-HA, Flag-Ubr1^{C1220S}) are specific. A further control was done to prove whether Ubr1-HA binds unspecifically to the IgG Sepharose beads using an *Δubr1 Δfas1* strain expressing Ubr1-HA from the same high-copy plasmid. As can be seen in Figure 23A (Lane 10) no Ubr1-HA could be detected with HA antibody in the eluate. This is in contrast to the finding in chapter 5.3.6 where little amount of Ubr1-HA was detected (see Figure 22A, Lane 6). Two more washing steps of the IgG beads prior treatment with TEV protease (see chapter 4.4.8) explain most likely this discrepancy.

5.3.7 Interaction of orphan Fas2 and Ubr1 is not interrupted in the absence of Ssa chaperones

A mammalian E3 ligase, called CHIP (carboxy terminus of Hsc70-interacting protein), was found to act as co-chaperone of Hsp70 and Hsp90, promoting the degradation of chaperone-bound immature substrates (Arndt et al, 2007; Murata et al, 2001). In detail, CHIP binds via its TPR (tetratricopeptide repeat) domain with the carboxy terminus of cytosolic and nuclear chaperones Hsc70 and Hsp90. With the help of an E1- and an E2-enzyme, CHIP mediates polyubiquitination of a chaperone-bound non-native protein, triggering the turnover of the chaperone client. As degradation of orphan Fas2 is dependent on the Hsp70 chaperone Ssa1 (see chapter 5.2.1) and the E3 RING ligase Ubr1 (5.3.2 – 5.3.4) it raised the question whether the association of orphan Fas2 and Ubr1 (see chapter 5.3.5 and 5.3.6) is bridged by the Ssa1 chaperone. To answer this question the interaction of orphan Fas2 and Ubr1 was analyzed in a strain expressing ssa1-45 as the sole source of a Ssa chaperone. Therefore TAP pull down experiments of Fas2-TAP using the *ssa1-45 Δssa2 Δssa3 Δssa4 Δubr1 Δfas1 FAS2-TAP* yeast strain carrying a high-copy plasmid encoding either HA-tagged wild type Ubr1 (Ubr1-HA) or the corresponding empty vector (negative control) were performed. To inactivate ssa1-45 function the culture was shifted from 25°C (permissive temperature) to 37°C (non-

permissive temperature) one hour before harvesting. As during heat treatment at 37°C proteins tend to aggregate and therefore become insoluble, especially when lacking Ssa chaperone activity, the pellet fraction obtained by centrifugation was also analyzed (see chapter 4.4.2 for more details). After native cell lysis it was checked whether Ubr1-HA could be co-purified with Fas2-TAP. Here, Fas2-TAP was released from the IgG beads by boiling them in urea loading buffer (see also chapter 5.3.5). As shown in Figure 24, Ubr1-HA was co-purified with Fas2-TAP (Figure 24, Lane 6 and 12) from extracts obtained from cells harvested at both temperatures (25°C and 37°C). This means that the interaction between orphan Fas2 and Ubr1 is not abolished at both temperatures in a strain deleted for the Ssa chaperones (Ssa2 to Ssa4) and carrying the conditional ssa1-45 mutant. Whereas almost no Ubr1 and Fas2 is detected in the pellet fraction at 25°C (Figure 24, Lane 2 and 5), increased amounts appear at 37°C (Figure 24, Lane 8 and 11) indicating that these two proteins tend to become more insoluble during the heat shock. Solubility of PGK remains unaffected during this heat shock as no material is detectable in the pellet fraction at 37°C (Figure 24, compare lane 2 and 8 as well as lane 5 and 11).

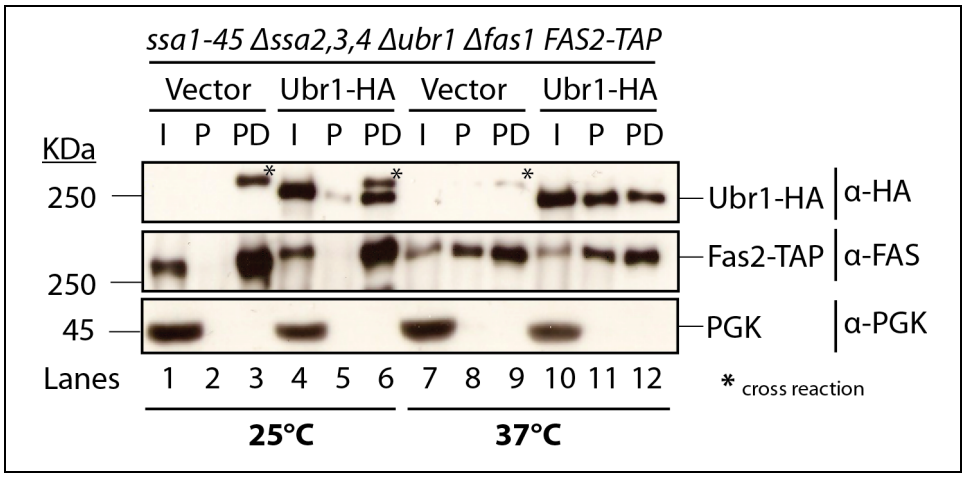

Figure 24. **Interaction between orphan Fas2 and Ubr1 is not dependent on Ssa chaperones.** High copy plasmid expressing HA-tagged wild type Ubr1 (Ubr1-HA) under the control of the *ADH1* promoter and the respective empty vector (negative control) were separately transformed into the *ssa1-45 Δssa2 Δssa3 Δssa4 Δubr1 Δfas1 FAS2-TAP* (YMS 146) strain. Cells were grown at 25°C and shifted, when needed, to 37°C for one hour before harvesting. Supernatant and Pellet obtained by native cell lysis and subsequent centrifugation at 16.200 g for 20 min were used as input (I) and pellet fraction (P), respectively. From the supernatant, Fas2-TAP was purified with IgG Sepharose beads and released by direct boiling in urea loading buffer (PD). All samples were subjected to SDS-PAGE followed by immunoblotting using HA-, FAS- and PGK antibodies.

5.3.8 Effect of additional E3 ligases than Ubr1 on orphan Fas2 quality control

As the degradation of orphan Fas2 was not completely blocked in a *Δubr1 Δfas1* strain (see Figure 17) the influence of other E3 ligases, which have been reported to be involved in cytosolic protein quality control (Fang et al, 2011; Heck et al, 2010; Metzger et al, 2008) were tested. The nuclear E3 ligase San1 has recently been identified to play an important role in cytosolic protein quality control (Heck et al, 2010; Prasad et al, 2010). In detail, some misfolded cytosolic proteins are imported into the nucleus for proteasomal degradation, involving San1 for polyubiquitination. As shown in Figure 25A, the proteolytical turnover of orphan Fas2 is somehow slightly affected in a *FAS1* deletion strain lacking the nuclear E3 ligase San1. Performing cycloheximide chases with different E3 mutants all deleted in *FAS1*, some small stabilization of orphan Fas2 in the *hul5 fas1* mutant (Figure 25A) was observed. To verify this finding pulse chase experiments with the same mutant strain were performed. In contrast to data obtained by the cycloheximide chase experiments given in Figure 25A, the degradation of orphan Fas2 in a pulse chase experiment is unaffected in a *HUL5 FAS1* deletion strain (Figure 25B). Given by former studies showing that the E3 ligase Doa10 is involved in the degradation of cytosolic proteins (Hwang et al, 2010; Metzger et al, 2008) cycloheximide chase analysis of orphan Fas2 in a *DOA10 FAS1* deletion was done. As shown in Figure 25A the degradation of orphan Fas2 was not affected in the absence of Doa10, indicating that the E3 ligase is not necessary for the elimination of the orphan substrate.

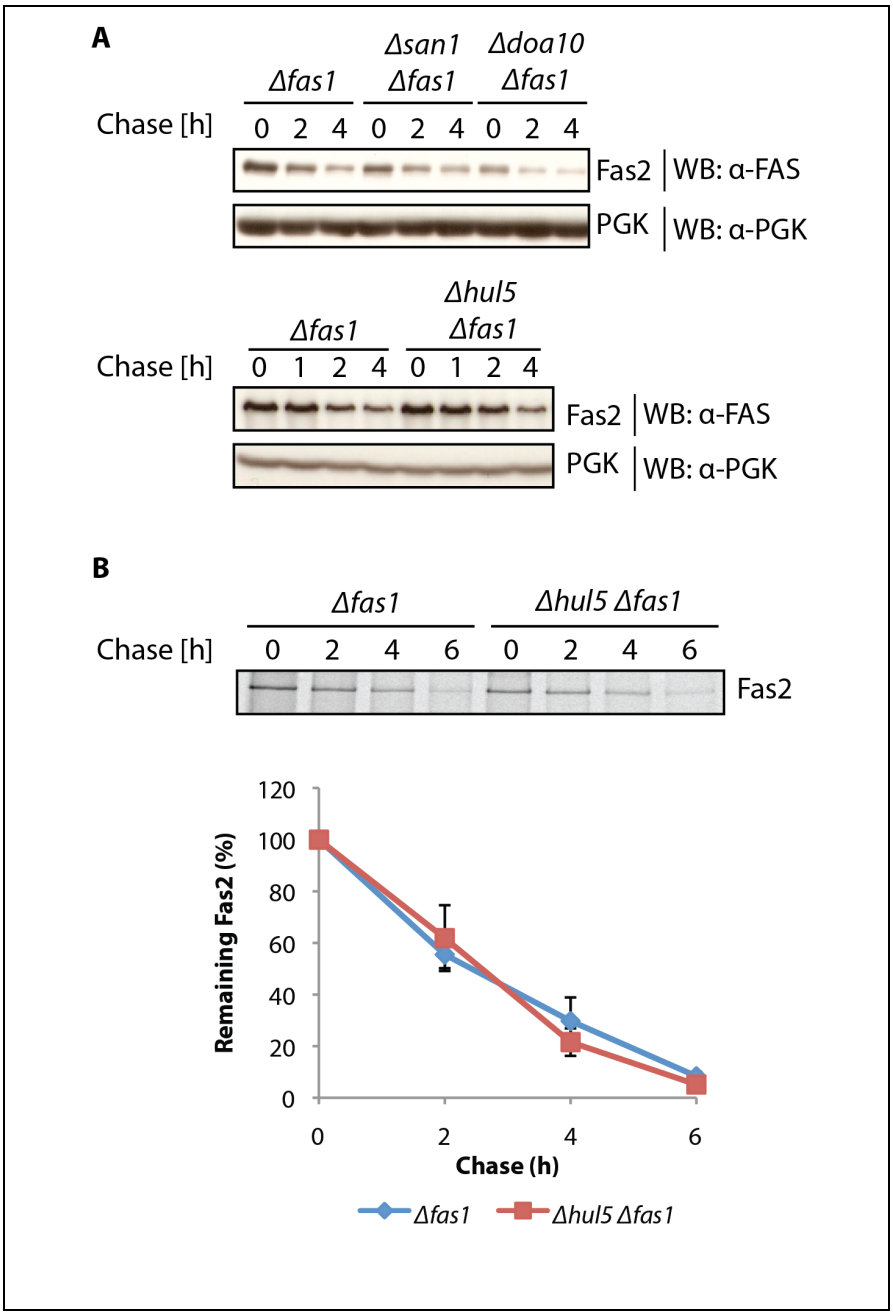

Figure 25. **Effects of other E3 ligases on quality control of orphan Fas2. A:** Cycloheximide chase analyses of orphan Fas2 in *Δfas1* (YMS 5)-, *Δsan1 Δfas1* (YMS 78)-, *Δdoa10 Δfas1* (YMS 73)- and *Δhul5 Δfas1* (YMS 176) cells were performed. Samples were collected at the indicated time points and subjected to Western blot analysis. Immunoblots were cut into two parts and incubated with FAS and PGK antibody, respectively. PGK served as loading control. **B:** Pulse chase analyses of orphan Fas2 with a *Δfas1* (YMS 5)- and a *Δhul5 Δfas1* (YMS 176) cells were performed. Samples were taken at the indicated time points. Fas2 was immunoprecipitated with FAS antibody, separated by SDS-PAGE and analyzed using PhosphorImager and Image Quant (Amersham Bioscience). Plotted data represent the mean values of three independent experiments. The error bars represent the standard deviation of the mean.

5.3.9 Cytosolic San1 reconstitutes orphan Fas2 degradation in the absence of Ubr1

When deleting the nuclear localization sequence (NLS) of San1, the E3 ligase is (mis)-localized to the cytosol (I. Amm, unpublished data). It was found that this mislocalized version of San1, (San1^{-NLS}), is able to restore the degradation of cytosolic misfolded model proteins in a *Δubr1* mutant (I. Amm, unpublished data). Hence, it was interesting to see whether San1^{-NLS} is also able to re-establish orphan Fas2 turnover in a *Δubr1 Δfas1* mutant.

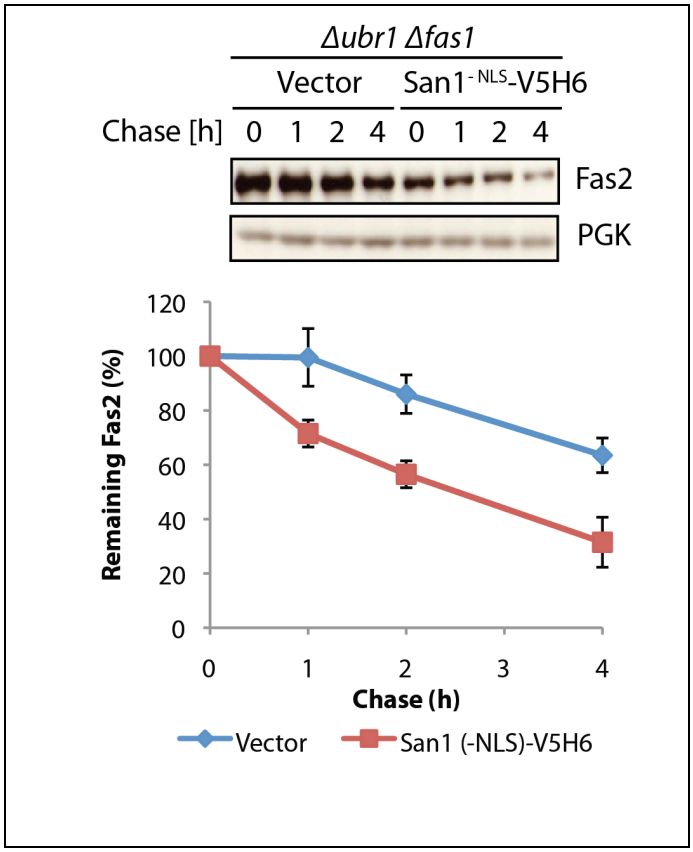

Figure 26. **Effect of cytosolic located San1 (San1-NLS) on orphan Fas2 quality control. A:** Cycloheximide chase analyses of orphan Fas2 in a *Δubr1 Δfas1* (YMS 23) deletion strain expressing either San1^{-NLS}-V5H6 under the control of the *GAL1* promoter from a plasmid or harbouring the corresponding empty vector were performed Cycloheximide was added (t=0h) to exponentially grown cells, samples were collected at the indicated time points and subjected to SDS-PAGE, followed by immunoblotting. Immunoblots were cut into two parts and incubated with FAS- and PGK antibody, respectively. PGK served as loading control. Band intensities of Fas2 were quantified with ImageJ and plotted as graph using Excel. Plotted data represent the mean values of two independent experiments. Error bars represent the standard deviation of the mean.

A plasmid expressing C-terminally V5-His6-tagged San1^{-NLS} under the control of the *GAL1* promoter was transformed into a *Δubr1 Δfas1* strain. The corresponding empty vector, transformed into a separate mutant, served as negative control. To monitor the proteolytically fate of orphan Fas2 in the presence or absence of San1^{-NLS}-V5H6, cycloheximide chase experiments were performed according to the protocol described in chapter 4.4.5 with the exception that the growth media contained 2% of galactose (for San1^{-NLS}-V5H6 expression). As seen in Figure 26, whereas degradation of orphan Fas2 is strongly delayed in the *Δubr1 Δfas1* mutant carrying the empty vector, the turnover of the orphan substrate is restored when San1^{-NLS}-V5H6 is expressed at the same time. This indicates that cytosolically located San1^{-NLS}-V5H6 is able to restore degradation of orphan Fas2 in a *Δubr1 Δfas1* mutant.

5.4 Involvement of the AAA ATPase Cdc48 in orphan Fas2 quality control

Besides its well characterized role in ERAD (Stolz & Wolf, 2010) recent findings have shown that the AAA ATPase Cdc48 is also necessary for the regulated degradation of two native cytosolic proteins, fructose-1,6-bisphosphatase (FBPase) and phosphoenolpyruvate carboxykinase (PEPCK) (Barbin et al, 2010).

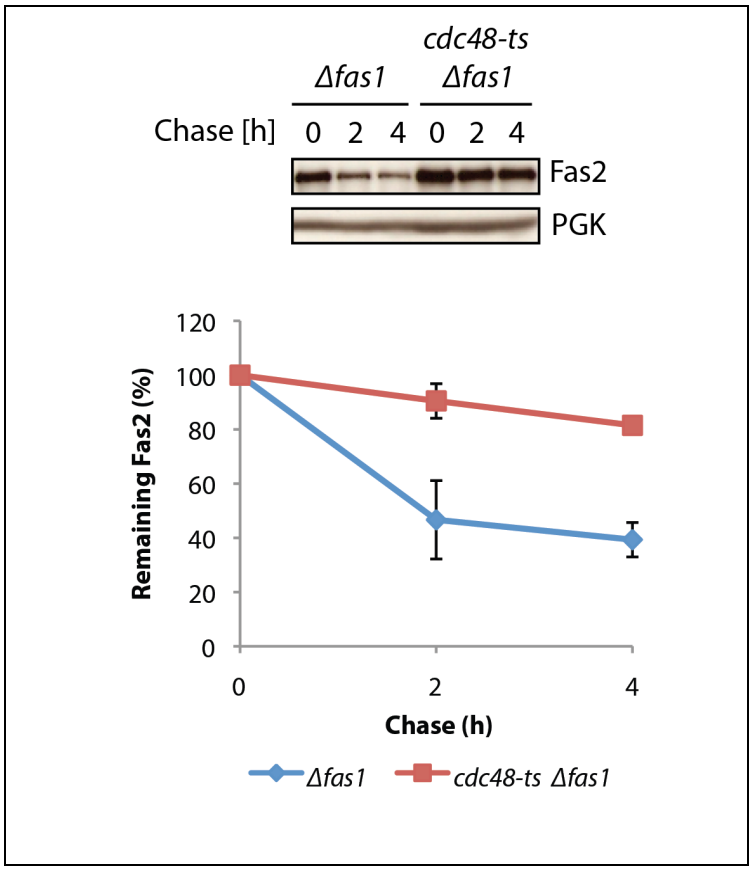

Figure 27. The AAA ATPase Cdc48 is required for orphan Fas2 degradation. Cycloheximide chase experiments of orphan Fas2 in *Δfas1* (YMS 5)- and *cdc48^{T413R} Δfas1* (YMS 45) cells were performed. Briefly, all strains were grown in YPD-FA media at 30°C until they reached the exponential growth phase. Cycloheximide was added (t=0h) one hour after incubation at 37°C. Samples were collected at the indicated time points. Immunoblots were cut into two parts and incubated with FAS- or PGK antibody. PGK served as loading control. Band intensities of Fas2 were quantified with ImageJ and plotted as graph using Excel. Plotted data represent the mean values of two independent experiments. Error bars represent the standard deviation of the mean.

The FBPase enzyme is stabilized, but still polyubiquitinated, in a strain carrying either a temperature sensitive allele of Cdc48 (*cdc48*^{T413R}) or Ufd1 (*ufd1-1*) or Npl4 (*npl4-2*) at restrictive conditions, indicating that the Cdc48-Ufd1-Npl4 complex acts after the polyubiquitination step of the substrate. Thus, it was suggested that upon polyubiquitination of the FBPase tetramer, catalyzed by a multi-subunit E3 ligase (GID complex), the Cdc48-Ufd1-Npl4 complex is required for the segregation of the polyubiquitinated FBPase tetramer from this GID complex, as well as for the dissociation of the tetramer into FBPase monomers. In addition it was proposed that Cdc48 is needed to create "loose ends" to make the FBPase monomers available for proteasomal degradation. According to these proposed functions of the Cdc48-Ufd1-Npl4 complex in FBPase degradation and the finding that most of the orphan Fas2 proteins are probably organized as homohexamers (see chapter 5.1.4) it raised the question whether Cdc48 is also required for the degradation of orphan Fas2. By using the same temperature sensitive mutant (*cdc48*^{T413R}), additionally deleted in *FAS1*, the turnover rate of orphan Fas2 in a cycloheximide chase experiment (see chapter 4.4.5) was monitored. As can be seen in Figure 27, degradation of orphan Fas2 was strongly impaired in the *cdc48-ts* mutant strain, indicating that Cdc48 is needed in the degradation process of orphan Fas2.

6 Discussion

Biological active proteins are essential macromolecules for cellular life as they are involved in a variety of cellular processes. However, mistaken, non-active proteins, including terminally misfolded or partially unfolded proteins, unassembled subunits of protein complexes ("orphan proteins") or proteins mislocalized to cellular compartments can pose a threat on cell homeostasis and cell vitality because they, or their correspondingly formed soluble or insoluble aggregates, could most likely interact with other proteins or cell components, disturbing their functionality. The cell toxicity of such mistaken proteins and protein aggregates is associated with the development of many neurological disorders including Alzheimer disease, Parkinson disease, and Huntington disease (Tyedmers et al, 2010). Therefore, cells possess protein quality control systems that are able to distinguish between properly folded proteins and unfolded or misfolded proteins. Components of these quality control systems will refold the latter or, when they are terminally misfolded, subject them to degradation. The components of protein quality control systems are molecular chaperones and the ubiquitin proteasome system. Molecular chaperones recognize hydrophobic patches of proteins, including newly synthesized or misfolded proteins and thus promote their folding into the native structure and prevent their aggregation (Bukau & Horwich, 1998; Cyr et al, 2002; Esser et al, 2004; Frydman et al, 1994; Hartl, 1996; Hohfeld et al, 2001; McClellan et al, 2005; Park et al, 2007; Young et al, 2004). When proteins do not reach their three-dimensional native structure, even with the assistance of molecular chaperones, they are considered as terminally misfolded proteins and are degraded by the ubiquitin proteasome system. Some studies showing also an involvement of molecular chaperones in the delivery of polyubiquitinated proteins to the proteasome for degradation (Cyr et al, 2002; Esser et al, 2004; Hohfeld et al, 2001; McClellan et al, 2005; Park et al, 2007). In contrast to the well-described protein quality control mechanisms of the ER limited knowledge is available for the protein quality control machinery acting in the cytosol.

The goal of my study was focussed on the cytosolic protein quality control of an unassembled protein subunit, the orphan Fas2 of the fatty acid synthase complex. When a protein complex is assembled it can be assumed that not always the exact amount of required protein subunits is synthesized or all synthesized protein subunits are indeed built into the complex. As excessive amounts of unassembled protein

subunits can cause the above-mentioned proteotoxicity in the cell, the orphan Fas2 protein must be eliminated by intracellular disposal systems. It has been reported that the 26S proteasome is necessary for the degradation of unassembled Fas2 (Egner et al, 1993). Therefore, I was very interested to identify additional components being involved in the recognition and elimination of this naturally occurring orphan protein. In addition I tried to reveal the mechanisms of these newly identified components acting in the quality control process of orphan Fas2. The study should also elucidate the question if there are different components in the recognition and elimination of so-called misfolded "model"-substrates of the cytosol which are usually not present in this environment (Park et al. 2007; McClellan et al, 2005) as compared to natural substrates of the respective compartment as is orphan Fas2.

6.1 Phenotypic characterization of the yeast *FAS1* deletion mutant

In yeast wild type cells most of the Fas2 subunits are assembled into fatty acid synthase macromolecules, making it difficult to monitor degradation of the unassembled species (see Figure 3). To overcome this difficulty I used a *FAS1* deletion strain where consequently all expressed Fas2 proteins must be considered as unassembled proteins (orphan Fas2). Because long-chain fatty acid synthesis is essential for cellular life yeast cells carrying a *FAS1*- or *FAS2* deletion are not viable in yeast standard media (YPD, CM). To cultivate such mutants yeast standard media were supplemented with myristic acid, which was solved in Tween 40. While yeast wild type cells, grown in YPD or YPD+FA, have a doubling time of about 90 minutes the *Δfas1* mutant exhibits a doubling time of 180 minutes. Media containing different ratios of different fatty acids (myristic acid, plamitic acid and stearic acid) was unable to increase the growth rate of the *Δfas1* mutant. It is known that for the uptake of exogenously provided fatty acids, which allow cellular growth of *Δfas1*, *Δfas2* and *Δole1* mutants of yeast on fatty acid supplemented media, requires the activity of several acyl-CoA synthetases, including Faa1-Faa4 and Fat1, as well as the serine/threonine kinase Ypk1 (Henry et al, 2012). However, the reason for the prolonged doubling time of the *Δfas1* mutant is unknown. In the case of fatty acid containing CM media, small amounts of yeast extract had to be added (so-called YCM+FA media) to overcome the observed slow-growth phenotype of the *Δfas1* mutant in CM+FA media (own observation). This

finding shows that the CM+FA media does not contain certain nutrient(s) which is/are required for proper growth of the yeast *Δfas1* deletion mutant.

6.2 Characterization of the Fas2 orphan protein

The fully assembled FAS complex has a molecular weight of about 2.6 MDa and is composed of six Fas2 and six Fas1 subunits forming a barrel-shaped dodecamer (Lomakin et al, 2007) (see also Figure 3). With an *in vivo* half-life of more than 20 hours the yeast cytosolic FAS complex is a long-lived, housekeeping enzyme. In the absence of Fas1, the *in vivo* half-life of the remaining Fas2 orphan protein decreases to about 2 hours. Whereas the long-lived FAS enzyme and, in case of a *Δfas2* deletion strain, orphan Fas1 are degraded within the vacuole, orphan Fas2 (*Δfas1* deletion strain) is targeted to the proteasome for elimination (Egner et al, 1993; Schuller et al, 1992). I was able to confirm the proteasome dependent turnover of orphan Fas2 using the proteasomal thermosensitive mutant *cim3-1* (see Figure 5). Protein degradation in the vacuole is mainly dependent on two prominent vacuolar proteinases, named Proteinase A (gene: *PEP4*/*PRA1*) and Proteinase B (gene: *PRB1*). Using the corresponding proteinase mutant (*Δpep4 Δprb1*) it was shown that elimination of orphan Fas2 is unaffected in the absence of the two proteinases (see Figure 5). This is in accordance with the results from Egner et al., 1993 and confirms the finding again that orphan Fas2 is exclusively targeted to the 26S proteasome for degradation. While the yeast fatty acid synthase is exclusively located to the cytosol (Huh et al, 2003) the eukaryotic 26S proteasome can be found in the cytosol and in the nucleus (Peters et al, 1994). Due to recent studies showing the import of misfolded cytosolic proteins into the nucleus for proteasomal degradation it was important to know whether elimination of orphan Fas2 occurs also in the nucleus or whether it remains in the cytosol. To address this question localization studies in wild type- and *Δfas1* cells were performed expressing C-terminally EGFP tagged Fas2 from its chromosomal locus. The same strains, expressing untagged Fas2 (no EGFP) were used as negative control. To visualize the nucleus cells were treated with the fluorescent dye Hoechst 33342. According to the localization pattern of the EGFP (green)- and Hoechst 33342 (red) fluorescence, shown in Figure 6, orphan Fas2-EGFP is, like Fas2-EGFP, only localized throughout the cytosol, indicating that the orphan protein is not imported into the nucleus for proteasomal degradation. It cannot be completely ruled out that the non-tagged orphan Fas2 (without EGFP) is

targeted to another cellular compartment than the cytosol. However, as Fas2 contains no specific targeting signal sequence and exhibits a very large size (207 KDa) it can be assumed that orphan Fas2 is neither targeted to another specific cell compartment nor imported into the nucleus. As orphan Fas2 is a naturally occurring protein without any mutation it was interesting to know whether it is a tightly folded or a loosely folded protein. To determine its folding state an *in vitro* trypsinization assay according to Prasad et al., 2010 was applied. In contrast to properly folded proteins loosely folded and unfolded species are much more susceptible to degradation in the presence of active proteases (e.g. trypsin). According to this definition it turned out that orphan Fas2 is a considerably unstable protein, even in the absence of trypsin (Figure 4), which can be explained by the fact that the cellular vacuolar proteases are very active during glass bead mediated cell breakage using no protease inhibitors. Thus, the folding state of orphan Fas2 can be considered as being easily accessible to proteolysis. It is likely that the exposed hydrophobic regions of orphan Fas2, which are normally masked by Fas1 in the FAS complex, cause the hypersensitivity to trypsin and released cellular proteases during cell breakage by glass beads. Orphan Fas2 cannot be completely unfolded, as it has obviously retained its ability to form the homohexameric Fas2 precomplex (Figure 8: *Δfas1*). In contrast, the fatty acid synthase and the 3-phosphoglyceratkinase (PGK) are considered as tightly folded proteins as they remain stable in cell extracts treated with trypsin (Figure 4).

The question whether orphan Fas2 exists as monomer or it is organized as a Fas2 homohexameric complex, as it is the case in wild type cells (Lomakin et al, 2007), was addressed by performing a glycerol step density gradient centrifugation with a native cell extract obtained from a *Δfas1* strain. Most of the orphan proteins were found in the high molecular mass fraction, ranging between more than 540 KDa and less than 2.6 MDa, indicating that orphan Fas2, with a molecular mass of 207 KDa, is most likely organized in assembly intermediates, which include Fas2 homohexamers. Very little amount of orphan Fas2 material was detected in the low molecular mass fraction (about 240 KDa), showing that very few orphan proteins exist as monomers. It is not clear, whether these monomers are products of disassembled Fas2 homohexamers, or whether they have not yet assembled. Taken all above discussed results together I can characterize orphan Fas2 so far as follow: (1) orphan Fas2 is proteolytically susceptible and (2) with an *in vivo* half-life of approximately 2 hours, orphan Fas2 is a substrate of the 26S proteasome, (3) orphan Fas2 is localized to cytosol, (4) most of the Fas2 orphan

proteins are organized in high molecular mass assembly intermediates, most likely as Fas2 homohexamers.

6.3 Cellular protein quality control of the Fas2 orphan protein

In the last years several cellular components have been identified playing an important role in the quality control process of cytosolic misfolded model proteins. These include different chaperones from different chaperone families and numerous components of the ubiquitin proteasome system. Only a few studies have been done to investigate the quality control process of physiological orphan proteins (Hill & Cooper, 2000; Lam et al, 2007; McClellan et al, 2005; Woods & Lazarides, 1985). In order to identify new components that are involved in the proteasome-mediated turnover of orphan Fas2 the degradation of the orphan protein in different yeast mutants, lacking specific chaperone activities or specific components of the ubiquitin proteasome system, was monitored using cycloheximide chase- and pulse chase experiments. It turned out that elimination of orphan Fas2 is dependent on the Hsp70 chaperone Ssa1 (see Figure 8). Ssa1 was found to be essential for degradation of some misfolded ERAD substrates, such as Pma1*, Ste6*, CTG* and unassembled Vph1 (Han et al, 2007; Hill & Cooper, 2000; Loayza et al, 1998; Taxis et al, 2003). It was further shown that the elimination of several cytosolic misfolded model substrates (Metzger et al, 2008; Park et al, 2007; Prasad et al, 2010) and an orphan protein (McClellan et al, 2005) were also dependent on the Hsp70 chaperone Ssa1. In addition our lab has recently revealed that the Hsp70 chaperone Ssa1 is also required for the proteasome-mediated degradation of two physiological cytosolic non-mutated proteins, FBPase and PEPCK (Juretschke, 2009). In the above-mentioned studies different functions for Ssa1 in protein degradation have been found or proposed. On one hand the Hsp70 is known to recognize hydrophobic patches of misfolded or unfolded proteins keep them in a soluble state or, when already aggregated, facilitate resolubilization. On the other hand it has been shown that the Hsp70 chaperone Ssa1 is able to promote polyubiquitination of certain substrates, indicating an active role of Ssa1 in protein degradation (Han et al, 2007; Needham et al, 2011). In a pull down assay (see Figure 24), I have noticed, that in the *ssa1-45* mutant strain, lacking in addition all other Ssa chaperones, orphan Fas2 is enriched in the pellet fraction (P) at non-permissive temperature (37°C). In contrast, no

Fas2 was detectable in the same strain background at permissive temperature (25°C). Here, the substrate was only detectable in the soluble fraction (I). Due to the lack of Fas1 subunits, which normally mask all exposed hydrophobic regions of Fas2 within the fatty acid synthase complex, it is likely that the different orphan Fas2 assembly intermediates (see Figure 6) are much more prone to form insoluble protein aggregates. I therefore suggest that the Hsp70 chaperone Ssa1 is needed to keep orphan Fas2 in a soluble state and to prevent the aggregation of the orphan substrate. However, it cannot be excluded that Ssa1 functions also in the delivery of the substrate to the proteasome. Hsp70 chaperone activities are promoted by different cofactors, including Hsp40 chaperones and NEFs. Whereas Hsp40 is responsible for making ATP binding and hydrolysis on Hsp70 efficient, NEFs are required to release ADP and thus the Hsp70-bound substrate. It was interesting to know which of the Hsp70 cofactors contribute to the quality control process of orphan Fas2 and therefore the proteolytic turnover of the orphan protein in corresponding mutant strains was monitored. A prominent Hsp70 cofactor involved in CytoQC is the Hsp40 chaperone Ydj1 (Lee et al, 1996; Metzger et al, 2008; Park et al, 2007; Prasad et al, 2010). Using a temperature sensitive allele of *YDJ1* (*ydj1-151*), surprisingly Ssa1-dependent degradation of orphan Fas2 was not affected under non-permissive conditions (37°C) (see Figure 10), suggesting that Ydj1 is not required for the elimination of the model substrate. This result is in contrast to the general view that the degradation of CytoQC substrates requires Ssa1 and Ydj1 (Prasad et al, 2010). However, it has been demonstrated that Ydj1 is also not needed for the degradation of a heterologously expressed orphan protein in the yeast cytosol, named VHL (McClellan et al, 2005), and the gluconeogenic enzyme FBPase (Juretschke, 2009). As the two orphan proteins (VHL and Fas2) and the FBPase monomer are natural proteins (without any mutation) it can be assumed that the folding of these substrates into a semi-stable/stable assembly-intermediate(s) conformation is more likely than to be recognized as strongly misfolded proteins and subsequently degraded by the proteasome. Reaching a certain folding state they are ready to undergo final assembly into corresponding proteolytically stable protein complexes (Fas2 into the FAS complex, VHL into VHL-ElonginBC complex, FBPase into FBPase tetrameric complex). Based on this view, the two orphan proteins (Fas2 and VHL) can be considered as not being strongly misfolded. Thus, it one may speculate that Ydj1 is rather required for the degradation of proteins that already during or directly after their synthesis are considered as being strongly misfolded mainly caused

by mutation(s) within their amino acid sequences. The Hsp40 chaperone Xdj1, which is closely related to Ydj1, and the Hsp40 chaperone Apj1, which was found to interact with Fas2 in wild type cells (Gong et al, 2009), are also not involved in orphan Fas2 degradation (see Figure 11 and (Schuster, 2010)). Thus, further Hsp40s have to be tested to find the corresponding candidate required for Ssa1 dependent degradation of orphan Fas2. Recent studies have demonstrated that the Hsp110 chaperones Sse1 and Sse2 play an important role in Ssa1-mediated degradation of certain cytosolic misfolded model substrates (Eisele, 2011; Heck et al, 2010; Prasad et al, 2010). It is assumed that the Sse chaperones mainly act as NEFs for Hsp70 chaperones, including Ssa1 and Ssb1 (Dragovic et al, 2006). However, an alteration of orphan Fas2 degradation in the absence of active Sse chaperones was not observed (Figure 12), indicating that other NEFs than Sse1 and Sse2 are required for Ssa1-dependent degradation of orphan Fas2. In contrast to Fas2, the Ssa1-dependent elimination of the VHL orphan protein requires the Sse1 chaperone activity as orphan VHL degradation is blocked in a *SSE1* deletion mutant (McClellan et al, 2005).

Most of the misfolded proteins used to study CytoQC exhibit a half-life ranging from a few minutes to about 30 min (Kaganovich et al, 2008; Metzger et al, 2008; Park et al, 2007; Prasad et al, 2010). However, the in vivo half-life of orphan Fas2 is approximately two hours and it was interesting to know whether this long half-life is due to a possible interaction with Hsp90 chaperones, which are known to act as so-called "holdases", promoting folding and complex assembly of their clients (Makhnevych & Houry, 2012), thus preventing them from rapid degradation (Caplan et al, 2007). It was speculated that during FAS biogenesis Hsp90 chaperones could keep Fas2 assembly intermediates as well as the Fas2 homohexamer in a FAS assembly-competent formation preventing its fast degradation. In order to prove this idea the Hsp90 chaperone function in a *Δpdr5 Δfas1* strain was inhibited by the drug geldanamycin. Then, the elimination rate of orphan Fas2 was carefully followed in a pulse chase experiment. According to the results shown in Figure 13, inhibition of the Hsp90 chaperones by geldanamycin do not lead to an accelerated degradation of orphan Fas2, indicating that the Hsp90 chaperones are not the reason for the moderate half-life of the model substrate. Either other factors protect orphan Fas2 from rapid proteasomal degradation and / or the Fas2 homohexamer itself is, at least *in vivo*, a relative stable complex. The Sti1 chaperone was identified to be essential for proteasome mediated elimination of orphan VHL (McClellan et al, 2005). However, no

alteration of orphan Fas2 degradation was observed in a *Δsti1 Δfas1* mutant strain (Figure 14). Finally a possible involvement of the Hsp104 chaperone was tested, which is able to unfold proteins (Glover & Lindquist, 1998; Lum et al, 2004), and the two sHsps, Hsp26 and Hsp42 were analyzed, which are found to bind unfolded- or partially unfolded proteins to prevent their aggregation (Friedrich et al, 2004; Stromer et al, 2004), in the quality control process of orphan Fas2. As shown in Figure 15, none of the above mentioned chaperones are required for the degradation of orphan Fas2.

Proteins destined for proteasomal degradation become, in most cases, tagged with polyubiquitin chains. Polyubiquitination of a substrate requires the sequential action of three enzymes, named E1, E2 and E3 (Hilt & Wolf, 2004). Recently, our lab identified the E3 ubiquitin ligase Ubr1 necessary for the elimination of the cytosolic misfolded model substrate ΔssCPY*-Leu2-13xmyc, which is an ER-import defective protein (Eisele & Wolf, 2008). Consequently, it was tested whether Ubr1 is also required for the degradation of orphan Fas2. As shown in Figure 17 and Figure 25A, the degradation of orphan Fas2 is indeed strongly delayed in the absence of the E3 RING ligase Ubr1. The half-life of Fas2 increases from 2 hours *(Δfas1)* to approximately 6 hours *(Δubr1 Δfas1)*. As degradation of orphan Fas2 is not completely blocked in a *UBR1* deletion mutant I tested the influence of ubiquitin ligases that have been reported to play also an important in degradation of cytosolic misfolded proteins (Fang et al, 2011; Heck et al, 2010; Khosrow-Khavar et al, 2012; Metzger et al, 2008). Neither the E3 RING ligase Doa10 nor the E4 HECT ligase Hul5 have shown an influence on the turnover of the Fas2 orphan protein (Figure 25). The nuclear E3 ligase San1, which has been reported to participate in degradation of, into the nucleus imported, cytosolic misfolded proteins, seems to have a very little effect on orphan Fas2 degradation (Figure 25A). Prior the import into the nucleus most of the nuclear proteins fold into their native conformation in the cytosol. Thus, it is possible that already properly folded San1 still located to the cytosol and on the way to the nucleus is able to participate to a low degree on the degradation of orphan Fas2. This idea is supported by the observation that a cytosolically mislocalized version of San1 (San1-NLS) is able to restore orphan Fas2 degradation in the absence of the E3 ligase Ubr1 (Figure 26). The HECT E3 ligase Ufd4 is most likely not involved in the degradation of orphan Fas2, as it has been shown that the turnover of the orphan substrate is unaffected in a strain lacking *UFD4* (Maxi Kanold, Diploma Thesis, 2010). Thus, other ubiquitin ligases might be involved in the elimination of orphan Fas2. It was not only interesting to identify the responsible

E3 ligase(s) required for orphan Fas2 degradation, but also to reveal the mechanism how the E3 ligase Ubr1 specifically recognizes this orphan protein. It is well known that Ubr1 is the N-recognin of the N-end rule pathway (Bartel et al, 1990; Varshavsky, 1997). The essence of the N-end rule is that the *in vivo* half-life of a protein depends on the identity of its N-terminal amino acid residue (for more details see chapter 3.2.2.1). Using different Ubr1 point mutants that are unable to degrade either type-1 or type-2 N-end rule substrates (Xia et al, 2008), it was observed that expression of a type-1 mutant of Ubr1 ($Ubr1^{D176E}$) in an *Δubr1 Δfas1* strain led to a strong stabilization of orphan Fas2. Expression of a type-2 mutant of Ubr1 ($Ubr1^{P406S}$) did not stabilize orphan Fas2 in the same strain background (Figure 19). These findings suggest that orphan Fas2 could be a putative type-1 N-end rule substrate. However, according to the Sherman rule the N-terminal methionine of Fas2 can not be cleaved off by methionine aminopeptidases (Map1 or Map2) because the side-chain of the second amino acid of Fas2, lysine, has a gyration radius of more than 1.29 Å (Moerschell et al, 1990). Thus, converting orphan Fas2 into a possible type-1 N-end rule substrate, an internal cleavage by one or more endopeptidases(s) must occur or a destabilizing type-1 amino acid has to be attached a the N-terminus of orphan Fas2. The RING domain of Ubr1 is required for ubiquitination of N-end rule- and non-N-end rule substrates (Heck et al, 2010; Khosrow-Khavar et al, 2012; Xie & Varshavsky, 1999). Failure in Ubr1-mediated substrate ubiquitination, caused e.g. by a catalytically inactive RING domain, leads to stabilization of the substrate. As shown in Figure 20 the degradation of orphan Fas2 is blocked in an *Δubr1 Δfas1* strain overexpressing an inactive RING mutant of N-terminally Flag-tagged Ubr1 ($Ubr1^{C1220S}$). In contrast, overexpressing N-terminally Flag-tagged wild type Ubr1 in the same strain background restores elimination of orphan Fas2. Based on these findings it seems most likely that Ubr1 is responsible for the ubiquitination of orphan Fas2. This assumption is supported by a study in which polyubiquitinated Fas2 species have been detected in yeast wild type cells (Peng et al, 2003). During the ubiquitin transfer E3 ligases usually bind their substrates or are at least in spatial proximity. To prove whether Ubr1 binds orphan Fas2 TAP pull down assays with a C-terminally TAP-tagged orphan Fas2 strain were performed, overexpressing C-terminally HA-tagged Ubr1 from a high copy plasmid. As shown in Figure 22, Ubr1-HA was indeed co-purified during pull down of Fas2-TAP, demonstrating that Ubr1 physically interacts with orphan Fas2. As discussed above, the type-1 site of Ubr1 is required for orphan Fas2 elimination (see Figure 19). However, binding of Ubr1-HA and orphan Fas2-TAP is not abolished when

Ubr1-HA is mutated in the type-1 substrate-binding site (Figure 23A). Similarly, the inactive RING mutant of N-terminally Flag-tagged Ubr1 (Flag-Ubr1^{C1220S}) is also still able to bind orphan Fas2 (Figure 23B). Taken together, whereas the RING domain and the N-end rule type-1 substrate-binding site of Ubr1 are not essential for binding of orphan Fas2 to the E3 ligase, they are required for its degradation. The type-2 substrate-binding site of Ubr1 is not critical, neither for binding (Figure 23A) nor for degradation (Figure 19) of orphan Fas2. Only a few natural substrates have been identified for Ubr1 (Byrd et al, 1998; Madura & Varshavsky, 1994; Rao et al, 2001). I am able to present a new physiological substrate of the E3 ligase Ubr1, the orphan protein Fas2. Furthermore, this study shows for the first time that the E3 RING ligase Ubr1 is involved in the quality control process of a naturally occurring orphan protein. Further studies have to be done to clear the question whether orphan Fas2 is processed to an N-end rule substrate, e.g. by endoproteolytic cleavage of the N-terminus revealing a type-1 destabilizing amino acid residue or if this orphan protein is recognized by Ubr1 in a way distinct from the N-end rule pathway. Studies with the mammalian cytosolic E3 ligase CHIP (carboy terminus of Hsc70-interacting protein) have revealed that this ligase binds to the C-terminus of Hsc70 and directs an ubiquitin-charged E2 enzyme to the Hsc70-bound client protein, which will be subsequently polyubiquitinated, followed by its proteasomal degradation (McDonough & Patterson, 2003; Pratt et al, 2010). Thus, Hsc70 has a bridging function in the interaction of the Hsc70-bound client protein and the E3 ligase CHIP. As orphan Fas2 degradation is dependent on the Hsp70 chaperone Ssa1 and the E3 RING ligase Ubr1, it was interesting to know whether a similar complex does also exist for Ubr1-Fas2-Ssa1. Therefore, ability of Ubr1 to bind orphan Fas2 in a strain deleted for the Ssa chaperones (Ssa2 to Ssa4) and carrying the thermosensitive *ssa1-45* allel was tested. As shown in Figure 24, overexpressed Ubr1-HA was co-purified under non-permissive conditions (37°C) upon pull down of chromosomally expressed Fas2-TAP in an *ssa1-45 Δssa2 Δssa3 Δssa4 Δubr1 Δfas1* mutant strain. This indicates that already existing interactions of orphan Fas2 and Ubr1 do not need the Ssa proteins Ssa2 to Ssa4 as well as a functional substrate-binding domain of Ssa1 (ssa1-45 carry a point mutation (P417) in the substrate binding domain). However, I cannot exclude that a structurally different region of the ssa1-45 mutant interacts with orphan Fas2. A significant alteration of orphan Fas2 degradation in different deletion strains lacking single E2 enzymes, Ubc2, Ubc4 (Figure 16) or Ubc8 (Maxi Kanold, Diplomarbeit, 2010) was not observed. However, double deletion of *UBC2* and *UBC4* leads to a strongly

delay of orphan Fas2 elimination (Figure 16). This indicates a complementing function of Ubc2 and Ubc4 in the quality control process of orphan Fas2. The question remains whether Ubr1 prefers one of the identified E2 for ubiquitination of orphan Fas2. The degradation kinetic of orphan Fas2 in a *Δubc2 Δubc4* strain is very similar to that found *Δubr1* mutant (see Figure 17), in which orphan Fas2's half-life increases from 2 hours to approximately 6 hours. An equal Ubc2-Ubc4 dependency has been found for Ubr1-mediated degradation of the transcription repressor Cup9 (Byrd et al, 1998) and a short truncated version of Gnd1 (stGnd1) (Heck et al, 2010). However, different E2 enzymes were found to be involved in Ubr1-dependent degradation of other model substrates, which were used to study CytoQC. For example, proteasome mediated elimination of cytosolic misfolded ΔssCPY*-Leu2-myc is already blocked in a *UBC2* deletion strain (Eisele, 2011), whereas degradation of a kinase subunit, Tpk2, is mainly dependent on Ubc2 and, to a minor portion on Ubc4 (Nillegoda et al, 2010). Ubr1 dependent degradation of the model substrate ΔssCG* is mainly triggered by the E2 enzymes Ubc4 and Ubc5 (Park et al, 2007). Further investigations have to be done to give an explanation why Ubr1 needs different E2 enzymes for the polyubiquitination of its client substrates. Our lab has recently found that the Cdc48-Ufd1-Npl4 complex is required for FBPase degradation, which occurs upon metabolic shift from ethanol to glucose (Barbin et al, 2010). Here, it was proposed that dissociation of the polyubiquitinated FBPase tetramer into FBPase monomer units is promoted by Cdc48-Ufd1-Npl4 complex. As I found that most of the orphan Fas2 proteins are organized as Fas2 homohexamers (Figure 8) the elimination of the orphan substrate in the absence of Cdc48 activity was tested. Indeed, cycloheximide chase experiments have shown that Cdc48 is involved in the degradation process of orphan Fas2 as the turnover of the substrate was blocked in a temperature sensitive mutant of Cdc48 at non-permissive conditions (see Figure 27). This result indicates that Cdc48 plays an important role in the cytosolic protein quality control of this naturally occurring orphan protein. It might be that Cdc48 is required for the segregation of polyubiquitinated Fas2 assembly intermediates into monomeric species (Barbin et al, 2010). Future studies should clarify which cofactors of Cdc48 are required for the proteasome-mediated turnover of orphan Fas2. Based on the results obtained in this study a hypothetical model of the cellular quality control for the orphan Fas2 protein is shown in Figure 28.

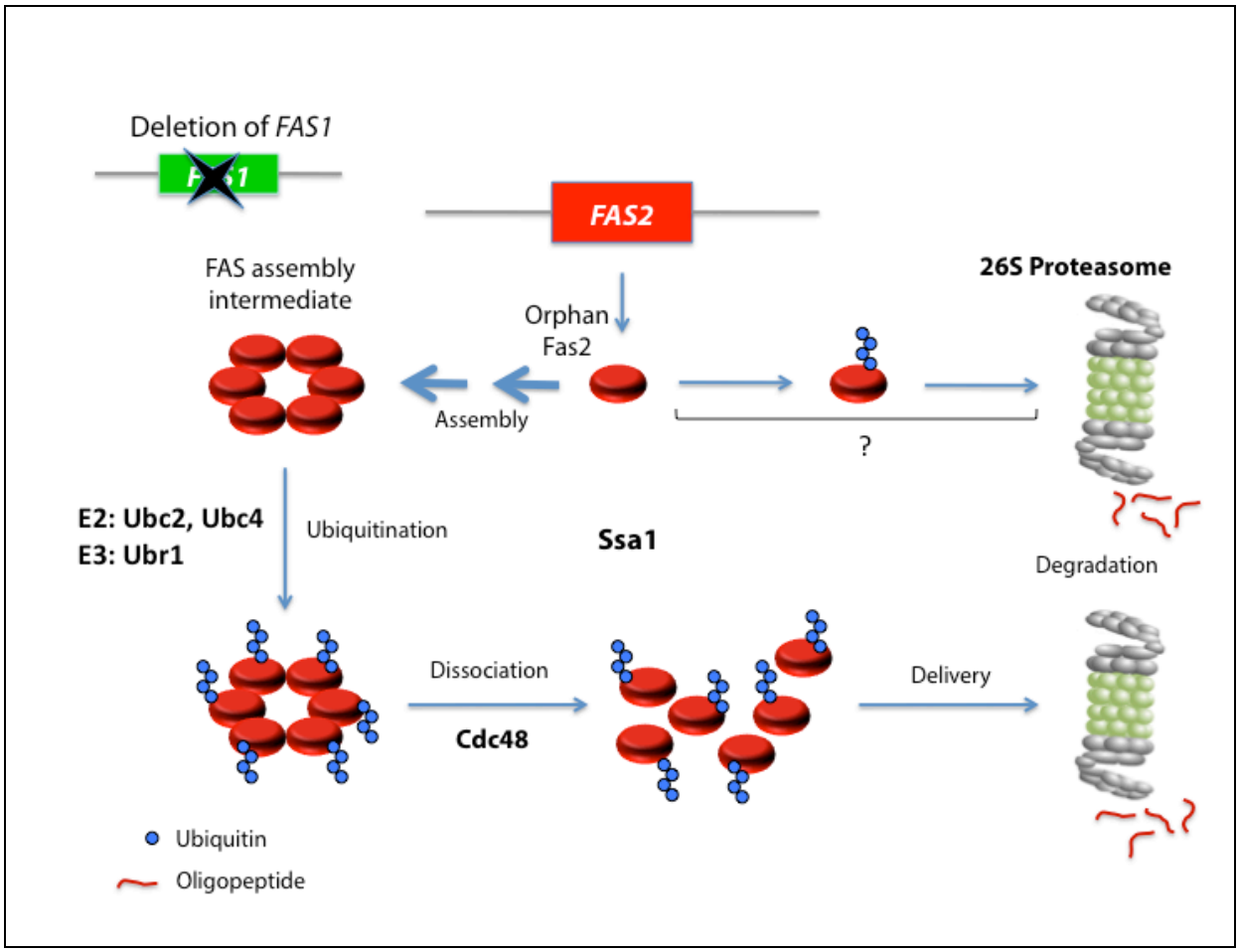

Figure 28. Hypothetical model illustrates the protein quality control of orphan Fas2.

The *FAS1*- and the *FAS2* gene encode the beta-subunit (Fas1) and alpha-subunit (Fas2), respectively, of the yeast fatty acid synthase. In the absence of the assembly partner Fas1 (*Δfas1*), Fas2 becomes an orphan protein. Orphan Fas2 is proteolytically susceptible and becomes a substrate of the 26S proteasome. Degradation of orphan Fas2 requires the Hsp70 chaperone Ssa1. It might be that Ssa1 keeps the orphan protein in a soluble state. Most of the orphan Fas2 proteins are organized in high-molecular assembly intermediates, consisting most likely of Fas2 homohexamers. Due to the absence of Fas1 subunits (*Δfas1*) final assembling of the FAS complex is not possible. Thus, the FAS assembly intermediate (Fas2 Homohexamer) is somehow recognized as unwanted protein complex. With the help of the cytosolic E2 enzymes Ubc2 and / or Ubc4, the Fas2 homohexamer is most likely polyubiquitinated by the cytosolic E3 RING ligase Ubr1. The AAA-ATPase Cdc48 is probably required for the dissociation of the polyubiquitinated assembly intermediate into monomeric Fas2 species. Finally, polyubiquitinated orphan Fas2 monomers are then targeted to the 26S proteasome for degradation.

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